Evidence for Psychosocial Stress-induced Inflammation, Altered Dopamine Status and Impaired Reward-directed Behaviour in Mice

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Summary

Stressful life events have been recognized as key proximal risk factors for depression, and exposure to stress is associated with altered immune function. Moreover, depression correlates with higher levels of inflammatory markers, at least in a subpopulation of patients. There is evidence that decreased dopamine (DA) neurotransmission in the mesocorticolimbic pathways leads to impaired reward and aversion processing and could underlie major psychopathologies, including motivational impairments that are common in depression. The psycho-neuro-immune hypothesis for depression presented here postulates that the inhibitory effects of stress-induced inflammatory signalling on DA neurotransmission represent a link between exposure to stressful environments and the emergence of depression-relevant behavioural abnormalities. Previous experiments have demonstrated that exposure to a chronic uncontrollable stressor – namely chronic social defeat (CSD) – increases fear learning-memory, helplessness and fatigue, and increases plasma levels of the pro-inflammatory cytokine TNF, in C57BL/6 young-adult male mice.

In this thesis I provide evidence that exposure to CSD activates the innate and adaptive immune system, alters DA transmission, and disrupts reward and punishment processing. Specifically, CSD led to increased splenic granulocytes, inflammatory monocytes and T helper 17 (Th17) cells, increased plasma levels of iNOS, and increased mRNA levels of kynurenine pathway enzymes in the liver. Moreover, microglia activation was evident in the ventral tegmental area (VTA). From the VTA, DA neurons project to different brain areas, including the nucleus accumbens (NAcc). In the NAcc, CSD mice showed decreased dopamine turnover (DOPAC/DA). CSD mice exhibited an attenuated hyper-locomotion response to a potent, selective DA transporter inhibitor. Behaviourally, CSD led to decreased operant responding for reward on a fixed-ratio schedule of reinforcement, and to reduced operant responses to an ambiguous reward stimulus in the learned non-reward (LNR) test, a behavioural assay for flexibility in the face of changing response-reinforcement contingencies.

To directly investigate the contribution of the DA mesolimbic system to the reward- and punishment-directed behaviours that are disrupted by CSD, I investigated the effects of 6-hydroxydopamine-induced DA depletion specific to the nucleus accumbens (NAcc) on mouse behaviour in the following test battery: reward motivation in a progressive ratio schedule (PRS) operant test, approach-avoid responding in a learned non-reward (LNR) operant test, footshock escape behaviour in a learned helplessness (LH) operant test, footshock avoid-escape behaviour in a treadmill operant test, and freezing behaviour in a Pavlovian fear conditioning test. NAcc DA depletion led to reduced responding: (i) for gustatory reward under effortful conditions in the PRS test; (ii) to a stimulus recently associated with gustatory non-reward in the LNR test; (iii) to footshock recently experienced as uncontrollable in the LH test; and (iv) to footshock that could only be avoided-escaped by physical effort in the treadmill test. Monoamine depletion was specific to DA and whereas 6-OHDA infusion into NAcc also led to a moderate DA reduction in prefrontal cortex, direct and specific DA depletion in the latter region did not impact on behaviour in the test battery used. Taken together, these novel mouse findings add significantly to the evidence that NAcc DA is a major regulator of motivational processing of both reward and aversion. They indicate the need for translational study of the targeting of pathophysiological DA function for the treatment of motivational psychopathologies in various psychiatric disorders.

In terms of antidepressant treatment, evidence indicates that the recently developed antidepressant agomelatine (Valdoxan), a melatonin receptor 1/2 agonist and serotonin receptor 2C antagonist, exhibits increased therapeutic efficacy relative to placebo in moderate-severe

depression. Moreover, agomelatine (AGO) exhibits greater efficacy in treating interest-pleasure deficits relative to venlafaxine. I investigated AGO effects on mouse CSD-induced disruption of reward-directed behaviour in a progressive ratio schedule (PRS) test, a probabilistic reversal learning (PRL) test, and in terms of home cage reward wanting and consumption. Oral administration of AGO led to moderate brain exposure to the compound at 1 h. In the PRS test, CSD decreased reward motivation and sub-chronic per os AGO led to a non-significant reversal of this effect. In the PRL test, CSD decreased reward-stay probability and the number of reversals achieved, and AGO reduced the latter effect. In the home cage, CSD mice exhibited decreased operant responding for saccharin reward and water; in terms of consumption, CSD mice exhibited decreased saccharin drinking during the active period whereas they increased water drinking during the inactive period. Sub-chronic per os AGO was without effect on each of these CSD changes in reward-directed and homeostatic behaviours. With respect to the AGO effects in the PRS and PRL tests, it is noteworthy that, via 5-HT_{2C} antagonism, AGO increases DA release in prefrontal cortex.

By combining environmental, neurochemical and pharmacological manipulations and investigating their effects on immune, neurochemical, cellular, molecular and behavioural readouts, this thesis provides mouse model evidence for the importance of stress-immune system-dopamine interactions in the aetiopathophysiology of the disrupted motivational processing that is common in psychiatric and immune disorders.

Zusammenfassung

Umfangreiche Studien haben gezeigt, dass Stress das Risiko einer Depression massgeblich erhöht. Stress übt einen modulatorischen Einfluss auf unser Immunsystem aus und auch eine Depression ist oftmals mit Zeichen einer Immunaktivierung assoziiert. Kernsymptome einer Depression sind eine beeinträchtigte Verarbeitung positiver und negativer Ereignisse und eine langanhaltende Lustlosigkeit, welche höchstwahrscheinlich auf eine Störung des dopaminergen Systems zurückzuführen sind. Das mesokortikolimbische dopaminerge System ist an der Steuerung des Belohnungs- und Motivationszentrums beteiligt. Gemäss der Psycho-Neuro-Immun-Hypothese übt die durch Stress ausgelöste Immunantwort einen inhibitorischen Einfluss auf das dopaminerge System aus und ist dadurch massgeblich an der Entstehung von Symptomen beteiligt, die auf eine Depression hinweisen. Chronischer Stress in C57BL/6 Mäusen, insbesondere sozialer Stress, führte zu verstärktem Angstlernen/-gedächtnis, Hoffnungslosigkeit und Erschöpfung. Zudem konnten erhöhte Plasmalevel des entzündungsfördernden Zytokins, TNF, in den Mäusen festgestellt werden.

Im Rahmen dieser Thesis möchte ich aufzeigen, dass chronisch sozialer Stress (CSD) sowohl eine angeborene als auch eine erworbene Immunantwort auslöst, die dopaminerge Transmission beeinflusst sowie zu einer beeinträchtigten Verarbeitung positiver und negativer Reize führt. Zeichen für eine Immunaktivierung in CSD-Mäusen sind eine erhöhte Anzahl inflammatorischer Monozyten und Typ17-T-Helferzellen, ein erhöhter Plasma-iNOS-Spiegel und ein Anstieg der mRNA der Kynurenin-Enzyme in der Leber. Zudem lässt sich eine Mikrogliaaktivierung in dem ventralen Tegmentum (VT) nachweisen. Neurone im VT projiezieren unter anderem zum Nucleus accumbens (NAcc). Der Dopaminumsatz im NAcc chronisch gestresster Mäuse (CSD) ist im Vergleich zu Kontrollmäusen deutlich reduziert. Des Weiteren dämpft CSD die Steigerung der Lokomotion in Folge einer einmaligen Gabe eines selektiven Dopamin-Wiederaufnahmehemmer. Verhaltenstechnisch führt CSD im „learned non-reward“-Test mit einem fixen Intervall zu einem beeinträchtigten Belohnungslernen für einen positiven als auch für einen nicht eindeutig positiven Verstärker.

Um den direkten Zusammenhang zwischen dem mesokortikolimbischen dopaminergen System und den veränderten Verhaltensmuster von CSD-Mäusen zu erforschen, untersuchte ich den Einfluss einer Dopamindepletion des NAcc mittels Hyrdoxydopamin auf das Verhalten der Mäuse anhand folgender Testbatterien: Einem operanten „progressive ratio schedule“-Test (PRS) für die Motivation, einem operanten „learned non-reward“-Test (LRN) für das Annäherungs-Vermeidungs-Verhalten, einem operanten „learned helplessness“-Test (LH) für das Fluchtverhalten in Folge eines Fussschocks, einem operanten Aktivitätstest für das Fluchtverhalten in Folge eines Fussschocks und einem Pawlowschen Angstkonditionierungstest.

Durch die Dopamindepletion im NAcc waren die CSD-Mäuse im PRS-Test weniger motiviert einen Aufwand für eine Belohnung in Form von Nahrung zu erbringen. Dies traf ebenfalls für einen Reiz zu, welchen die Mäuse mit einer nicht präferierten Nahrung assoziierten. Des Weiteren zeigten CSD Mäuse ein beeinträchtigtes Fluchtverhalten für einen unkontrollierbaren Fussschock im LH-Test als auch für einen, durch körperliche Leistung, kontrollierbaren Fussschock im operanten Aktivitätstest auf dem Laufrad. Die Dopamindepletion hatte keine Auswirkungen auf andere monoaminerge Neurotransmittersysteme, bewirkte jedoch eine leichte Abnahme der Dopaminfreisetzung im präfrontalen Kortex. Eine direkte Hydroxydopamininjektion in den präfrontalen Kortex hatte jedoch keinen Einfluss auf das Verhalten der Mäuse. Daraus lässt sich folgern, dass das dopaminerge System im NAcc massgeblich an der Regulation der Belohnungs- und

Bestrafungsverarbeitung beteiligt ist. Translationale Studien sind nun erforderlich, um die pathophysiologische Bedeutung von Dopamin in verschiedenen psychiatrischen Krankheiten zu verstehen.

Das erst kürzlich entwickelte Antidepressiva Agomelatine (Valdoxan) ist ein Melatonin-Rezeptor-Agonist und ein Serotonin-Rezeptor-2C-Antagonist und zeigt eine effiziente Wirkung in mittelschweren und schweren Fällen von Depression. Zudem ist Agomelatine (AGO) wirkungsvoller für die Behandlung der Anhedonie verglichen mit Venlafaxine. Im Rahmen meiner Thesis untersuchte ich die Wirkung von AGO auf das dysregulierte Motivations- und Belohnungsverhalten in CSD-Mäusen mit Hilfe eines „progressive ratio schedule"-Test (PRS) und einem „probabilistic reversal learning"-Test (PRL), als auch mit der Erfassung des Belohnungsstreben im Heimkäfig. Agomelatine wurde oral verabreicht. CSD reduzierte das motivationale Handeln von Mäusen im PRS- und PRL-Test. AGO war in der Lage diese Beeinträchtigung des Belohnungssystems zu normalisieren. Im Heimkäfig zeigten CSD-Mäuse ein reduziertes Verlangen nach Saccharin während der aktiven Phase, tranken jedoch mehr Wasser während der inaktiven Phase. AGO hatte keinen Effekt auf diese stress-induzierten Verhaltensänderung. Im Hinblick auf denn normalisierenden Effekt von AGO im PRS- und PRL-Test lässt sich sagen, dass Agomelatine, aufgrund seiner 5-HT2C antagonistischen Eigenschaften, zu einem erhöhten Dopaminfreisetzung im präfrontalen Kortex führt.

Mit Hilfe von ökologischen, neurochemischen und pharmakologischen Manipulationen und deren Einflüsse auf immunologische, neurochemische, zelluläre, molekuläre und verhaltensbezogene Systeme im Mausmodel verdeutlicht diese Arbeit die Bedeutung der Interaktion zwischen Stress, dem Immunsystem und Dopamin hinsichtlich der zugrundeliegenden Aetiopathophysiologie motivationaler Störungen in zahlreichen psychiatrischen und Immunkrankheiten.

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General introduction

1. Translational approach to depression research

Current clinical classifications systems (DSM-5, ICD-10) define major depressive disorder (MDD, hereon referred to as depression) as a mood disorder characterized by core symptoms of 1) depressed mood, with a focus on negative events and feelings of sadness and helplessness, 2) reduced interest-pleasure for daily life activities and 3) increased fatigability, as lack of energy and reduced activity. However, from a phenomenological point of view, depression is a very heterogeneous condition (Langenecker et al., 2014). Most likely, the observed phenotypical heterogeneity reflects the existence of multiple pathophysiological routes, targeting particular behavioural/cognitive domains (Webb et al., 2015). In support of this view, current psychiatric nosology has been accused to create "… an artificial grouping of heterogeneous syndromes with different pathophysiological mechanism into one disorder…" (Wong et al., 2010). To parse the clinical and pathophysiological heterogeneity of depression and develop rapid and effective treatment or prevention strategies for each individual patient, an experimental classification for mental disorders has been advocated (Insel, 2014; Wong et al., 2010).

The realization of a basic research-based classification system and the overcoming of the "disease entity" model (Cuthbert, 2015) has been the objective of the National Institute of Mental Health (NIMH) Research Domain Criteria (RDoC) initiative.

1.1 An RDoC-inspired framework for depression research

The RDoC project

The NIMH RDoC initiative was introduced in 2008 to "develop, for research purposes, new ways of classifying mental disorders based on behavioural dimensions and neurobiological measures" (Cuthbert, 2015). The RDoC project considers psychiatric diseases from a translational research perspective, supporting the development of a precision medicine approach for mental disorders (Cuthbert and Insel, 2013).

Current psychiatric nosology is based almost exclusively on presenting signs and symptoms, with classification systems for mental disorders (i.e. DSM-5, ICD-10) looking at disorders as symptom complexes based on clinical descriptions (Cuthbert, 2015). RDoC reverses the classic approach of starting with clinical definitions of mental disorders and seeking for biological correlates or risk factors (Cuthbert, 2015). An inventory of basic behavioural/cognitive functions and the related brain circuits is created and psychopathology is considered in terms of disruptions of the normal-range operation of specific primary behavioural functions and of the neural systems implementing these functions (Cuthbert and Insel, 2013). By doing so, the RDoC initiative operationalizes a fully dimensional approach to mental disorders (Dillon et al., 2014). There is indeed an explicit focus on the full range of functioning from normal to abnormal (Cuthbert and Insel, 2013; Woody and Gibb, 2015).

The RDoC project created a 'matrix' (Table 1) (http://www.nimh.nih.gov/research-priorities/rdoc/research-domain-criteria-matrix.shtml) comprising four major axes (Cuthbert, 2015). The first axis – the columns in the matrix – comprises behavioural domains and their constituent dimensions, as primary objects of study. Dimensions, or 'constructs', are grouped into five superordinate domains of function: Positive Valence Systems, Negative Valence Systems, Cognitive

Systems, Systems for Social Processes, and Arousal/Regulatory Systems. Each domain then includes several subordinate dimensions. The second axis – the rows in the matrix – consists of variables of study that are used to assess the dimensions. They are grouped into units of analysis and include genes, molecules, cells, circuits, physiology, behaviour, and self-reports. The third and fourth axes are neurodevelopmental trajectories and environmental influences, respectively, and their interactions are also important. The integration of clinical signs and symptoms as well as neurobiological measures for depression into an RDoC-informed framework could well assist in parsing the heterogeneity of depression as a clinical entity, and link different pathophysiological pathways to particular behavioural disturbances.

Table 1. The research domain criteria and their dimensions

Negative Valence Systems	**Positive Valence Systems**	**Cognitive Systems**	**Systems for Social Processes**	**Arousal and Regulatory Systems**
Acute threat ("fear")	Approach motivation	Attention	Affiliation and attachment	Arousal
Potential threat ("anxiety")	Initial responsiveness to reward	Perception	Social Communication	Circadian Rhythms
Sustained threat	Sustained responsiveness to reward	Declarative memory	Perception and Understanding of Self	Sleep and wakefulness
Loss	Reward learning	Language behaviour	Perception and Understanding of Others	
Frustrative nonreward	Habit	Cognitive (effortful) control		
		Working memory		

Adapted from Cuthbert and Insel (2013) and Cuthbert (2015)

Clinical and biological findings for depression need to be integrated into the RDoC classification system. The Positive Valence System and the Negative Valence System include core constructs for depression, and are the most heavily studied in depression research (Langenecker et al., 2014). Nonetheless, dimensions within Cognitive Systems, Systems for Social Processes, Arousal and Regulatory Systems are also relevant to depression.

Positive Valence System in depression

The Positive Valence System (PVS) includes approach motivation, initial responsiveness to reward, sustained responsiveness to reward, reward learning and habit, as major dimensions (Cuthbert and Insel, 2013). Diminished interest or pleasure is one of two symptoms required for a diagnosis of depression, along with depressed mood (DSM-5). Depressed individuals exhibit blunted responses to rewarding information, possibly representing a deficit in the approach-related or appetitive system (Bylsma et al., 2008). From these findings stems the hypothesis that depression is characterized by hyposensitivity to reward (Eshel and Roiser, 2010).

Indeed, blunted encoding of reward-related stimuli, reward-related decision making and reinforcement learning are core facets of depression (Pizzagalli, 2014). Current clinical diagnosis does

not discriminate between a decrease in motivation towards rewarding stimuli and a reduction in experienced pleasure (Treadway and Zald, 2011). To overcome this symptom heterogeneity, a refined definition of anhedonia has been proposed (Treadway and Zald, 2011), which takes into account the distinction between a diminished hedonic response to rewards (consummatory anhedonia) and a reduced motivation to pursue them (motivational anhedonia). Depressed individuals do not show impaired consummatory pleasure as measured by the Sweet Taste Test (Dichter et al., 2010), while they are less likely to mobilize high effort to obtain desirable outcomes (Sherdell et al., 2012).

Anhedonic individuals also show a deficit in adapting their behaviour as a function of previous reinforcements (Dillon et al., 2014). Indeed, depressed subjects fail to integrate reinforcement history into decision making (Dillon et al., 2014) when assessed in a probabilistic reward task (Pizzagalli et al., 2008). This suggests that reward learning is also impaired in depression (Pizzagalli et al., 2005). Thus, regarding the PVS, depression is characterized by reduced approach motivation (Sherdell et al., 2012) and reward learning (Pizzagalli et al., 2005) while initial responsiveness to reward lies in the normal range of functioning (Dichter et al., 2010).

Negative Valence System in depression

The Negative Valence System (NVS) includes dimensions responsible for responses to aversive situations or context, such as acute threat ("fear"), potential threat ("anxiety"), sustained threat, loss and frustrative nonreward (Cuthbert and Insel, 2013).

The NVS dimension, perceived sustained threat, also interfaces with behavioural dimensions that are dysfunctional in depression (Eshel and Roiser, 2010). Altered reactivity to aversive stimuli, including motivation to respond, contributes to the core depression symptoms of low mood and high fatigue, and involves various states that are common in depression (Eshel and Roiser, 2010). For example, experiencing lack of control of aversive stimuli can lead to helplessness, including reduced motivation to attempt control (Pryce et al., 2011b; Treadway and Zald, 2011); lack of control of an aversive stimulus can lead to increased fear conditioning to stimuli that predict the aversive stimulus (Nissen et al., 2010). Increased emotional reactivity to and decreased motivation to control aversive stimuli both contribute to high fatigue (Demyttenaere et al., 2005). Moreover, several lines of evidence point towards the presence of behavioural abnormalities related to the construct of loss in depression (Woody and Gibb, 2015). Behavioural alterations pertaining to loss include self-reports of hopelessness, rumination, psychomotor retardation and attentional bias to negative valenced information. Regarding the latter, in depressed subjects there is evidence for mood-congruent memory biases towards negative events (Gotlib et al., 2004; Watkins et al., 1992) and difficulties in classifying emotional content of facial stimuli (Langenecker et al., 2005). Thus, regarding the NVS, constructs of loss and sustained threat are central to depression, as evidence by the presence of memory/attentional biases (Gotlib et al., 2004; Watkins et al., 1992), sense of helplessness (Pryce et al., 2011b), increased punishment sensitivity (Eshel and Roiser, 2010), and psychomotor retardation and fatigue (Demyttenaere et al., 2005; Singh et al., 2013).

Therefore, depression can be considered in terms of a dysregulation or dysfunction of behavioural/cognitive dimensions spanning both the PVS and the NVS (Langenecker et al., 2014). Deconstructing the heterogeneity of depression symptomatology and the respective pathophysiological pathways is the route to identification of efficacious dimension-specific anti-

depressant strategies and the emergence of a precision medicine approach in psychiatry (Insel, 2014).

1.2 Depression-relevant behavioural readouts in rodents

Basic research and animal models are fundamental for the identification of genetic, molecular and neural substrates for defined behavioural dimensions as well as for the identification of specific etiopathophysiological pathways influencing behavioural disorders (Cuthbert and Insel, 2013). To enable increased understanding of reward and aversion processing and the changes that underlie depression, neuropsychological paradigms are required that 1) enable the quantitative measurement of these neuropsychological processes and their responses to genetic, environmental and pharmacological manipulations; 2) can be applied in depressed patients, healthy humans, and laboratory animals (Pryce and Seifritz, 2011). In this section, translational tests for the assessment of reward and punishment-related behaviours that are relevant for depression symptoms will be described. The tests presented were used throughout Study A, B and C.

Testing reward sensitivity in mice: relevance for PVS

As presented in section 1.1, reward involves several psychological components: liking (reactions to hedonic stimuli), wanting (motivation process of incentive salience), Pavlovian stimulus-stimulus learning and goal-directed learning (instrumental learning with cognitive representations) (Berridge, 2003; Berridge and Kringelbach, 2015). Reward consumption has been investigated in rodents using sucrose preference tests (Vialou et al., 2010; Wilkinson et al., 2011). Motivational components of reward have been assessed using operant behaviour tasks, e.g. progressive ratio schedule (Hamill et al., 1999; Ishiwari et al., 2004) and the concurrent fixed ratio/chow feeding choice task (Nunes et al., 2014; Nunes et al., 2013a). Paradigms for reward-related learning in mice include Pavlovian conditioned place preference (Pena et al., 2014), and the goal-directed test of probabilistic reversal learning (Ineichen et al., 2012) and learned non-reward (Nilsson et al., 2012).

Progressive ratio schedule test and operant sucrose-preference test

The progressive ratio schedule or reinforcement (PRS) test is used to assess subjects' motivation to obtain rewarding stimuli under effortful conditions (Hamill et al., 1999; Ishiwari et al., 2004). The PRS test is based on operant (response-outcome) learning, with mice first learning that a specific operant action (e.g. nose-poke into a stimulus hole) is followed by delivery of a reward-goal (e.g. sucrose pellets). Training for the PRS test is conducted on a fixed ratio (FR) 1 schedule; that is, one nose-poke leads to the delivery of one sucrose pellet. Within the course of a single PRS session, the requirement in terms of number of responses to access reward increases across trials. The animal initially emits a predetermined number of responses to obtain a reward (e.g., 1 response, start ratio = 1). Following reinforcer delivery, the subsequent response requirement increases by some increment (e.g., another 3 responses) such that in the next trial the animal is required to complete more responses than in previous trials (e.g., 4 responses before reward delivery) (Ineichen et al., 2012; Roane, 2008). The session can be terminated after a maximum session duration or a long time interval without response emission (break-point).

Assessment of willingness to exert an effort in order to obtain a reward has been conducted also in humans, using the effort expenditure for rewards task (EEfRT) (Barch et al., 2014). Using the EEfRT, (Treadway et al., 2012) reported that depressed individuals are less willing to exert physical effort to obtain potentially larger rewards compared with healthy probands.

The sucrose preference test is commonly used in rodents to measure reward consumption (Papp et al., 1991). In the sucrose preference test, animals are confronted with a two-bottle choice situation, with one bottle containing water and the other a sweet-tasting solution (e.g. saccharine); absolute values of water and saccharine consumption and percent saccharin preference are measured (Cathomas et al., 2015a). However, depressed patients do not show blunted hedonic impact in a human sweet taste test (for review see Treadway and Zald, 2011). To assess rodent motivation for gustatory rewarding stimuli that is continuously available in the home cage, an operant form of the classical sucrose preference test can be used (Cathomas et al., 2015a), namely the IntelliCage operant choice test. In an Intellicage (Krackow et al., 2010) equipped with operant doors that control access to water and sucrose-filled bottles, mice have to make an operant response (e.g. nose-poke) to open the door and access the bottles.

Both the PRS test and the IntelliCage operant choice test provide readouts for mouse motivation to obtain gustatory reward under effortful conditions.

Learned non-reward test

Adjusting behaviour in accordance to altered response-reward contingencies can be assessed in reinforcement-based paradigms, including reversal learning (Nilsson et al., 2012). Successful learning of such schedules is based on two main mechanisms: learned non-reward (LNR) – also referred to as the learned irrelevance – and perseveration. Perseveration refers to the tendency to continue to respond to a stimulus associated with negative feedback. Conversely LNR refers to reduced attention directed at a stimulus or a stimulus dimension that proves to be task irrelevant (Maes et al., 2006). The LNR test assesses the subject's behaviour in a two-choice discrimination test where the stimulus previously associated with punishment and is now associated with reward and a second stimulus is novel and associated with punishment. Behavioural performance in this test reflects the subject's sensitivity to punishment (negative feedback), measured in terms of ability to learn that a stimulus-punishment association has changed to a stimulus-reward association (Maes et al., 2011). Recently, (Nilsson et al., 2012) established a LNR test in mice. In the mouse LNR test, animals first learn a two-choice spatial discrimination (SD) task where a response in the correct nosepoke stimulus leads to the delivery of a single sucrose pellet and a response in the incorrect nosepoke stimulus does not. When the subject attains criterion at the SD stage, it enters the LNR stage: the previously correct nosepoke stimulus is removed, the previously incorrect nosepoke stimulus becomes correct, and a third, novel nosepoke stimulus, not available at the SD stage, is introduced as the new incorrect nosepoke stimulus. To learn correct responding, the subject must unlearn the stimulus-punishment association and learn that the same stimulus is now associated with reward. Healthy human probands commit more errors in a learned non-reward test than they do in a perseverance test (Maes et al., 2006). Interestingly, depressed humans commit more errors in an extra-dimensional shift test, which assesses both learned non-reward and perseverance, than do healthy subjects (Michopoulos et al., 2006). Thus, the LNR test represents an important translational tool to assess subjects' flexibility to changing response-reward contingencies.

Probabilistic reversal learning test

The probabilistic reversal learning task (PRL) test is based on two-way reversal learning: in two-way reversal learning, subjects are required to exhibit continuous responding to the operant stimulus that is associated with reward (win-stay responses), including after predictable reversals in the operant stimulus associated with reward (lose-shift responses). In PRL, a proportion of correct responses is not reinforced i.e. on such trials responding to the correct stimulus is punished in terms of omission

of reward plus a delay to the next trial. This allows for assessment of the subject's negative feedback sensitivity: the more frequently the subject chooses the incorrect stimulus (lose-shift) on the trial after unexpected negative feedback, the greater the subject's negative feedback sensitivity (NFS). NFS was increased in depressed patients relative to controls (Taylor Tavares et al., 2008) and in healthy volunteers following acute tryptophan depletion to reduce central 5-HT (Cools et al., 2008). It has been proposed that increased PRL-NFS in depression is due to high-amplitude transient-phasic 5-HT signalling of aversion (Cools et al., 2008). A study using a PRL test in rat has been published (Bari et al., 2010), and we have validated and studied 5-HT involvement in a PRL test in mouse (Ineichen et al., 2012).

Testing aversion sensitivity in mice: relevance for NVS

Fear conditioned freezing

Classical fear conditioning is a form of associative learning in which an animal acquires a defence response to a neutral conditioned stimulus (CS, e.g. light, tone) that is paired to an innately aversive unconditioned stimulus (US, e.g. painful foot electroshocks) (Blair et al., 2001). In rodents, fear can be measured as freezing behaviour to inescapable aversion. Fear acquisition leads to a progressive increase in the freezing behaviour. Associative fear learning and memory can then be tested by assessing the expression of freezing behaviour in response to the CS when the animal is subsequently exposed to it in the absence of the US (Blair et al., 2001). This form of fear conditioning and expression is associated with the synaptic plasticity changes in pathways of the lateral amygdala (AMYG) (Blair et al., 2001).

The human version of the fear conditioning paradigm uses visual or tone CS and electroshock US, and skin conductance, which increases due to sweating induced by activation of the sympathetic autonomic nervous system, to measure emotional responsiveness to the CS (Nissen et al., 2010). Interestingly, Nissen et al. (2010) showed enhanced fear conditioning in depressed patients compared with healthy control subjects. Classical fear conditioning is a behavioural assay that can be used to measure generalized increased emotionality in response to aversive stimuli both in humans and in rodents (Azzinnari et al., 2014).

Learned helplessness

Generalized learned helplessness (LH) comprises the experiencing of an uncontrollable stressor leading to a generalized perception of aversive events as being uncontrollable, even if they are not (Pryce et al., 2012). The concept of LH was initially introduced in rodent behavioural research (Anisman and Merali, 2001). Animals receive either controllable aversive stimuli, e.g. electroshocks that can be terminated by an operant response, or uncontrollable aversive stimuli of the same intensity and duration. Then, both groups are tested and compared with controllable aversive stimuli, e.g. in the two-way shuttle arena where moving to the opposite site of the arena and avoiding or escaping electroshock is the control response (Pryce et al., 2012). The animals that experienced uncontrollable electroshocks, compared to those that experienced controllable electroshocks, exhibit a deficit in escapes in the test phase (Pryce et al., 2012; Pryce and Seifritz, 2011).

Human LH tests have been adapted from the animal paradigms and typically also use inescapable versus escapable electroshocks. LH has been proposed to be a major aetiological process influencing vulnerability to and onset of depression (Abramson et al., 1978; Pryce et al., 2011b). Moreover, LH

has been proposed as a major psychopathological mechanism in maintenance of depression (Pryce et al., 2011b). Interestingly, increased helplessness during and after exposure to uncontrollable aversive stimuli has been shown in depressed patients relative to healthy controls (Diener et al., 2009; Strigo et al., 2008). Thus, LH is a behavioural assay to measure generalized impaired cognitive control and decreased motivation to react to aversive stimuli that can be applied both in humans and in rodents (Azzinnari et al., 2014).

Physical fatigue in an aversive environment (Treadmill test)
The state of fatigue is a core symptom of depression, which is at the interface of somatic, emotional and cognitive pathologies (Pryce and Seifritz, 2011). Fatigue can be divided into components of psychomotor retardation, physical tiredness and mental fatigue (Demyttenaere et al., 2005). In rodents, fatigue has been tested by measuring activity on a running wheel (Ifuku et al., 2014) or running to avoid-escape an escapable footshock on a treadmill (Azzinnari et al., 2014). In the treadmill test mice are first assessed in their ability to run away from an escapable footshock at a lower speed; on the following day avoid-escape capability is tested under more effortful conditions (Azzinnari et al., 2014).

In humans, the physical tiredness component of fatigue has been measured using the measurement of grip strength on a dynamometer (Shiratori et al., 2014). Interestingly, depressed patients exhibit rapid reduction of the grip strength of the right forelimb across trials of maximum grip strength (Emerson et al., 2001). Human fatigue is commensurate with decreased motivation and perceived ability to maintain a level of physical or mental effort (Hossain et al., 2003; Keller et al., 2007; Weinstein et al., 2010). Thus, fatigue tests can be used to assess motivation and exertion of effort towards rewards or away from aversive stimuli.

1.3 The role of stress in depression
Stressful life events have been recognized as key proximal risk factors for depression (Kendler et al., 1999; Slavich et al., 2009) influencing the development, expression and exacerbation of the disease (Brown and Harris, 1978; Lora and Fava, 1992). When stressful events are associated with depression, they typically occurred within 3-6 months prior to depression onset (Kendler et al., 1998). Moreover, chronic life stressors are associated with higher probability of relapse occurrence (Lethbridge and Allen, 2008), more severe depressive symptoms (Leskela et al., 2006) and higher treatment resistance (Amital et al., 2008).

Several authors have investigated whether some classes of life events are more likely to induce depression (for review see Hammen, 2005). Among chronic psychosocial stressors, experiences that are characterized by perceived lack of control of the aversive situation, interpersonal loss (e.g. loss of status, humiliation) and induce “psychobiological programmes of defeat and submission” (Gilbert, 1992) have high depressogenic capabilities (Kendler et al., 2003). Uncontrollability is certainly a major characteristic of each of these life-event dimensions (Pryce et al., 2011b). Emphasis on the uncontrollability of stressors is consistent with reports showing that perceived control over stressful events is a key modulator of physiological stress responses, with uncontrollable stressors being associated with larger cortisol changes (Dickerson and Kemeny, 2004).

Although stressors influence the occurrence of the first episodes of depression, they are less linked to recurrences (Daley et al., 2000; Kendler et al., 2000). These findings support the kindling/sensitization hypothesis in depression (Post, 1992), which postulates that stress and

previous depressive episodes can sensitize affected subjects, thus increasing the risk for future depressive episodes, even in the absence of stressors.

Effects of stress on PVS and NVS behaviours in rodents

As stated in section 1.2, animal research is fundamental for further understanding of depression aetiopathophysiology. The establishment of valid animal models for depression not only requires behavioural assays of relevance to depression neuropsychopathology but also the use of genetic and/or environmental manipulation(s) of aetiological relevance to depression (Pryce and Seifritz, 2011).

Given the role of environmental stressors as risk factors in depression aetiology, various forms of chronic stressors, involving repeated applications of an uncontrollable and unpredictable stress, have been used as a manipulation to induce depression-relevant behavioural states in rodents, primarily in mice (Krishnan and Nestler, 2011). These stressors include social isolation (Schiavone et al., 2012), social instability in the form of alternate phases of isolation and crowding (Herzog et al., 2009), chronic unpredictable mild stress (CUMS) (Jayatissa et al., 2008; Papp et al., 2003), repeated social disruption (Wohleb et al., 2012), and chronic social defeat (CSD) (Azzinnari et al., 2014; Krishnan et al., 2007). Findings of preclinical studies using the stress models presented here show that exposure to chronic stressors induces dysfunctions in behavioural domains that pertain to both the PVS and NVS.

Regarding the effects of stress on reward-related behaviours, it has been shown that prolonged social isolation during adulthood results in reduced sucrose drinking and alterations in sexual reward behaviour (Wallace et al., 2009), and that social instability, CUMS and CSD reduce sucrose intake (Herzog et al., 2009; Krishnan et al., 2007; Papp et al., 2003) and place-preference conditioning i.e. reduced approach to a place previously paired with a reward (Papp et al., 1991). However, the findings of reduced preference for palatable stimuli after stress exposure – usually referred to as anhedonia-like behaviours – points towards a deficit in the behavioural domain of initial responsiveness to reward rather than of reward motivation and learning. Given that depression is characterized by impaired reward motivation (Sherdell et al., 2012) and learning (Pizzagalli et al., 2005), while initial responsiveness to reward lies in the normal range of functioning (Dichter et al., 2010), stress models assessing reward-directed effort expenditure and reward learning are advocated. In rats, Jaisinghani and Rosenkranz (2015) showed that stress does not affect operant responding for rewards under a fixed ratio (FR) 4 schedule, and higher ratios or PR schedules have not been tested. Interestingly, when rats were assessed in a PRS test, stress reduced operant responding for rewards (Jaisinghani and Rosenkranz, 2015).

The effects of stress on 1) reward-directed motivation and 2) cognitive flexibility to changing response-reward contingency will be addressed in Study B and C, respectively.

Regarding the effects of stress on punishment-related behaviours, it has been shown that chronic psychosocial stress in mice increases contextual and cued fear conditioning (Yu et al., 2011) and induces a negative response bias (Papciak et al., 2013), suggesting increased expectation of aversive events (Enkel et al., 2010). Moreover, early life stressors increase sensitivity to conditioned place aversion (Ventura et al., 2013). Our laboratory recently showed that CSD in mice induces increased fear acquisition, decreased 2-way avoid-escape responding (increased helplessness) and increased fatigue (Azzinnari et al., 2014)

2. Neurobiology of NVS and PVS

The psychological-behavioural processes that constitute NVS and PVS and mediate individuals' responses to aversive and rewarding stimuli, respectively, share a common neural substrate: the mesocorticolimbic dopamine (DA) system (for review see Bromberg-Martin et al., 2010; Salamone and Correa, 2012).

2.1 The Dopamine system: Neuroanatomy and Neurochemistry

Dopaminergic pathways

Most dopamine (DA)-synthesizing neurons in the brain are located in either the midbrain substantia nigra pars compacta (SNpc) or the ventral tegmental area (VTA) (Sesack and Grace, 2010). Projection axons arising from the SNpc and VTA follow defined pathways via the medial forebrain bundle (MFB) to innervate specific subcortical and cortical structures (Hattori, 1993). The nigrostriatal pathway projects from the SNpc to the dorsal striatum and has a prominent role in execution and planning of movement (Gepshtein et al., 2014) and in motor flexibility (Bestmann et al., 2015). Moreover, the nigrostriatal pathway plays an important role in non-motor functions (McClure et al., 2003), with striatal tonic DA release coordinating processes including working memory, implicit learning, decision making and planning (Brooks, 2006).

The mesocortical pathway arises in the VTA and projects to frontal and temporal corticies, specifically the anterior cingulate and prefrontal cortex (Ford and Williams, 2008). This pathway is important for executive functions such as working memory (Floresco, 2013). The mesolimbic pathway also arises in the VTA and projects to the ventral striatum (including the nucleus accumbens, NAcc), bed nucleus of the stria terminalis, hippocampus, amygdala, and septum (Ford and Williams, 2008; Sesack and Grace, 2010). It is particularly important for the regulation of reward motivation (Salamone and Correa, 2012; Salamone et al., 2007). The NAcc is part of the ventral striatum and has extensive connectivity with other brain regions. It receives excitatory inputs from the PFC and the amygdala, and inhibitory afferents from the ventral pallidum (VP). Major projection regions of the NAcc include the VP and, reciprocally, the VTA (Sesack and Grace, 2010).

Dopamine signaling

In DAergic neurons, the amino acid tyrosine is converted into DA by tyrosine hydroxylase (TH). TH is the rate-limiting enzyme for the biosynthesis of the catecholamines dopamine, noradrenaline, and adrenaline (Dickson and Briggs, 2013). After synthesis, DA is transported into synaptic vesicles through the vesicular monoamine transporter type 2 (VMAT2) (Narendran et al., 2015).

Dopamine exerts its effects on postsynaptic neurons by binding with DA receptors. DA receptors are divided into 2 classes: the DA 1 (D1) family (comprising the D1 and D5 subtypes) and the DA 2 (D2) family (comprising the D2, D3, and D4 subtypes) (Dalley and Everitt, 2009). Both classes are G-protein-coupled receptors and are distinguishable by an opposite modulation of adenylate cyclase. The DA D1-like receptor family positively regulate adenylate cyclase activity to increase intracellular cyclic AMP (cAMP), while the D2-like class of receptors is negatively coupled to adenylate cyclase and inhibits the synthesis of cAMP (Dalley and Everitt, 2009). These opposite effects on cAMP result in an opposite regulation of protein kinase A (PKA) activity by D1 and D2 receptors. After release into the extracellular space, DA is cleared from the synaptic cleft by uptake into presynaptic nerve terminals by the dopamine transporter (DAT).

Biological effects of DA on target neurons depend on the post-synaptic signaling elicited by DA receptors. One major molecular target for the actions of DA is the DA- and cAMP-regulated phosphoprotein, Mr 32kDa (DARPP-32) (Fienberg et al., 1998; Zachariou et al., 2006). DARPP-32 is enriched in dopaminoceptive neurons (Zachariou et al., 2006) and is a crucial mediator of the biochemical, electrophysiological, transcriptional, and behavioural effects of DA (Svenningsson et al., 2004). Upon PKA phosphorylation at Thr-34, DARPP-32 behaves as a potent inhibitor of protein phosphatase 1 (PP1) (Hemmings et al., 1984). Conversely, phosphorylation of DARPP-32 at Thr-75 by cyclin-dependent kinase-5 (Cdk5) converts it into an inhibitor of PKA (Bibb et al., 1999). Thus, both D1 and D2 intracellular signal transduction pathways converge on DARPP-32 (Tretter and Gebicke-Haerter, 2012), that acts as an integrator for DA transmission (Svenningsson et al., 2004).

2.2 Contribution of the DAergic mesocorticolimbic system to behavioural regulation

The mesocorticolimbic DA system has been shown to modulate behavioural responses to both rewarding and aversive stimuli (Salamone and Correa, 2012). Dopamine neurons in the VTA that project to the NAcc are integral to these functions, with NAcc DA signalling serving to modulate the responsiveness of NAcc neurons to excitatory and inhibitory inputs, and thereby to modulate behavioural activation and inhibition by environmental stimuli (Alexander et al., 1990).

DA system and sensitivity to rewards

Much of the evidence for DA function in NAcc is derived from loss-of-function studies using pharmacological DA depletion or antagonism. Regarding the former, infusion of the monoamine neurotoxin 6-hydroxydopamine (6-OHDA) into the NAcc, leading to specific degeneration of DA axons efferent to NAcc and also DA cell body death in VTA, is a well-established method (Aberman and Salamone, 1999). It is known that NAcc DA depletion does not affect food consumption (Cousins et al., 1994) and that NAcc DA signaling does not regulate emotional “liking” reactivity to rewards (Smith et al., 2011). However, DA depletion in rat NAcc impairs acquisition of and rate of responding on operant schedules of reward reinforcement, particularly under conditions requiring high effort (Aberman and Salamone, 1999), including PRS tests (Hamill et al., 1999). Moreover, NAcc DA depletion delays development of sucrose preference (Martinez-Hernandez et al., 2012). These findings indicate that NAcc DA directly regulates motivation for rewarding stimuli (Nunes et al., 2013b). In addition to the importance of NAcc DA in regulating reward motivation, as characterised in rat, there is also increasing interest in the involvement of DA in the modulation of behavioural flexibility under conditions of changing reward contingency (Cools et al., 2011; van Schouwenburg et al., 2010). Studies in nonhuman primates showed that NAcc DA is responsible for encoding the incentive properties of stimuli and Pavlovian reward prediction error (Schultz, 1998). Consistent with animal data, human functional magnetic resonance imaging (fMRI) studies have described robust activation in the ventral striatum (NAcc) in response to rewards (O'Doherty, 2004) and this neural activation is stronger during the anticipation, rather than the consumption, of rewards (Dillon et al., 2008). Accordingly, the ventral striatum has been strongly implicated in encoding reward prediction error and the hedonic value of outcomes, and it is robustly recruited during reward anticipation (Pizzagalli, 2014).

DA system and sensitivity to aversive stimuli

Mesocorticolimbic DA also modulates punishment-related behaviour. Indeed, DA is acutely released in the NAcc in response to painful stimuli (Young et al., 1993) and social stress (Cabib and Puglisi-Allegra, 2012). Mesocorticolimbic DA also modulates the acquisition and expression of Pavlovian conditioned fear (Pezze and Feldon, 2004) and of punishment active avoidance (Wenzel et al., 2015).

For example, in rats, depletion of NAcc DA leads to a deficit in operant lever pressing to avoid-escape electric footshocks (McCullough et al., 1993), and in DA-deficient mice where DA expression could be reactivated region-specifically, only restoration of DA signalling to both the entire striatum and the amygdala enabled mice to learn two-way active avoidance (Darvas et al., 2011). In rats, midbrain DA depletion increases sensitivity to inescapable footshocks, i.e. reduces responding in a lever-press escape test (learned helplessness) (Winter et al., 2007). Moreover, electrophysiology studies in rats and rhesus macaques have demonstrated that DA neurons respond to punishment and its anticipation (Bromberg-Martin et al., 2010). Interestingly, a recent pharmacological fMRI study performed in healthy humans showed that the DA synthesis enhancer levodopa increased striatal activation by punishing stimuli (Wittmann and D'Esposito, 2015), supporting the hypothesis that DA contributes to punishment processing.

2.3 DA system dysfunction in depression

As stated above, the DA mesocorticolimbic system controls a number of psychological processes pertaining to both the NVS and the PVS. These include reward-directed motivation and reward-directed effort expenditure, reward learning, reactivity to and control of aversive environments, and fatigue. Concomitantly, depression can be interpreted as a dysfunction of the same behavioural domains that are influenced by the mesocorticolimbic DA system. DA dysfunction might, therefore, be important in the pathophysiology of depression (Dunlop and Nemeroff, 2007; Pizzagalli, 2014).

One of the first indications for reduced DA transmission in depression was reports showing that the levels of homovanillic acid (HVA), one of the major metabolites of DA, are reduced in the plasma and cerebrospinal fluid of depressed subjects (Mitani et al., 2006; Roy et al., 1985). Lambert et al. (2000) directly measured CNS HVA levels in the internal jugular vein using catheters. Reduced levels of HVA were found, providing a strong indication for reduced DA metabolites in depression. Moreover, post-mortem studies have provided evidence for reduced levels of the DA metabolite dihydroxyphenylacetic acid (DOPAC) in the caudate nucleus (Bowden et al., 1997), and reduced DAT levels in striatal regions (caudate, putamen, NAcc) of depressed subjects (Klimek et al., 2002). Consistent with reduced DAT levels, using positron emission tomography (PET), (Martinot et al., 2001) showed reduced striatal (^{18}F)-fluorodopa uptake in depressed patients with psychomotor retardation, providing a link between dopamine hypofunction and psychomotor retardation in depression.

Transient catecholamine depletion has been used in humans to assess a potential causal link between reduced DA levels and depression (Pizzagalli, 2014). Administration of a tyrosine hydroxylase inhibitor (α-methylparatyrosine, AMPT) can induce relapse of depressive symptoms in subjects with a history of depression (Berman et al., 2002; Hasler et al., 2004; Miller, 1996). PET studies have shown that AMPT-induced DA depletion affects reward-related brain regions receiving strong catecholamine innervations, including the OFC (Bremner et al., 2003). Stimulation of DA release with psychostimulants (e.g. dextroamphetamine) has been used to probe the DA system in depression. Depressed patients display reduced activation in reward-related brain regions (OFC, caudate, putamen) in response to pleasant stimuli, after receiving dextroamphetamine (Tremblay et al., 2005). These fMRI findings support the hypothesis of a hypofunctional DAergic reward system in depression (Tremblay et al., 2005).

The effects of stress on the DA system

On the basis of the observation that chronic stressors increase vulnerability to depression in humans (Kendler et al., 2000), preclinical research in animal models of depression investigated the neurobiological effects of prolonged exposure to stressors (Pizzagalli, 2014). Given the hypothesis that depression might stem from a hypofunctional DAergic system, a number of studies have explored the effects of stress on DA signaling. It has been show that exposure to chronic unavoidable stressors leads to DA system dysfunctions that include down-regulation of mesolimbic DA pathways (Cabib and Puglisi-Allegra, 2012), reduced DAT levels (Brake et al., 2004), and sensitization of mesocortical DA responses to subsequent stressors (Cuadra et al., 1999).

Exposure of rats to CUMS leads to increased threshold (reduced sensitivity) for operant self-stimulation of the VTA (Moreau et al., 1992), facilitates a DA response to aversive stimuli while blunting the DA response to rewarding stimuli (Di Chiara et al., 1999), and reduces basal striatal DA activity (Bekris et al., 2005). Interestingly, CUMS-induced DA blunting can be reversed by treatment with the antidepressant imipramine (Bekris et al., 2005). Moreover, other forms of inescapable chronic stressors (e.g. footshocks) can reduce the number of spontaneously active VTA DA neurons (Moore, 2001) and suppress basal and cocaine-induced DA NAcc transmission (Gambarana et al., 1999; Mangiavacchi et al., 2001). A biological marker indicative of blunted mesolimbic DA release is the decreased DAT levels in efferent regions of the mesocorticolimbic DA pathway. In rats, reduced DAT levels have been observed in the striatum (including the NAcc) after exposure to early life stress (Brake et al., 2004), chronic psychosocial stress (Lucas et al., 2004) and immobilization stress (Lucas et al., 2007). Moreover, rats bred selectively for reduced motivation for reward also showed reduced DAT (Jiao et al., 2003). Interestingly, reduced DAT levels have been reported in depressed patients (Klimek et al., 2002; Martinot et al., 2001). As mentioned in section 2.1, DARPP-32 is a crucial mediator of the behavioural effects of DA signaling (Svenningsson et al., 2004; Zachariou et al., 2006). Jin et al. (2015) have shown that CSD in mice increased expression of total DARPP-32, p-Thr34 DARPP-32, and p-Thr75 DARPP-32 in the PFC or AMYG. Potentiated/sensitized mPFC DA release in response to a novel stressor has been demonstrated in rats previously exposed to chronic stress (Chrapusta et al., 1997; Cuadra et al., 1999). Since one proposed effect of activation of mesocortical DA neurons is hypothesized to be their inhibition of NAcc and its activation by DA (King and Finlay, 1997), the stress-induced sensitization of mesocortical DA neurons might contribute to the maintenance of the depressive state. A sensitized mesocortical DAergic system could serve as a neurobiological correlate of the behavioural sensitization hypothesis for depression formulated by Post (1992).

3. The psycho-neuro-immune hypothesis for depression

A number of studies indicate that immune and inflammatory processes contribute importantly to the aetiopathophysiology of brain diseases, including neurodegenerative disorders, mood and anxiety disorders and schizophrenia (Haroon et al., 2012; Miller et al., 2013). With regards to depression, several lines of evidence indicate that inflammation is important at least in a significant sub-population of affected subjects (Haroon et al., 2012). The psycho-neuro-immune hypothesis for depression presented here postulates that activation of the immune system upon exposure to stressful environmental conditions impairs mesolimbic DA transmission, affecting the neural circuits that modulate behavioural responses to rewarding and aversive stimuli.

3.1 Immune system activation in depression

The first findings showing that depression is characterized by inflammation and monocytic and T cell activation were provided by Maes et al. (1990). This evidence for increased inflammatory biomarkers in depression, including increases in the production of inflammatory cytokines and increased cellular markers of immune activation (Maes et al., 1992), laid the foundation for a novel hypothesis that inflammation and cell-mediated immune activation are key factors in depression aetiopathophysiology (Maes, 2011). A substantial literature has reproduced and confirmed these findings, and meta-analytical studies have revealed that some of the most reliable peripheral biomarkers of increased inflammation in depression are increases in the pro-inflammatory cytokines interleukin (IL)-6, tumor necrosis factor (TNF)-α, and the acute-phase reactant, c-reactive protein (CRP), in the peripheral blood and the cerebrospinal fluid (CSF) (Haroon et al., 2012; Miller et al., 2009a). Activation of nuclear factor kappa B (NF-κB), a primary transcription factor in the initiation of the inflammatory response, has also been demonstrated in depressed patients (Lukic et al., 2014). Although much of the interest in inflammation and depression has been focused on the innate immune response, findings of altered lymphocyte activation markers (Euteneuer et al., 2012; Grosse et al., 2015; Miller, 2010) in depressed patients led to the hypothesis that both acquired (e.g., T and B cell) and innate (e.g., macrophage) immune responses may be involved (Miller, 2010; Miller et al., 2009a). Increased peripheral levels of inflammatory markers have been reported to correlate with the severity of depression-relevant symptoms, including fatigue, cognitive dysfunction, and impaired sleep (Bower et al., 2002; Meyers et al., 2005; Motivala et al., 2005). A complex interaction exists between peripheral inflammation and antidepressant treatment. Thus, whereas increased inflammatory markers normalize in depressed patients following successful antidepressant treatment (Miller et al., 2009a), depressed subjects that do not respond to antidepressant therapy also have increased inflammatory markers (Fitzgerald et al., 2006). The findings of reduced treatment response in patients with increased baseline inflammatory markers suggest a relationship between inflammation and treatment resistance (Fitzgerald et al., 2006; Lanquillon, 2000).

Several studies have investigated whether depression is associated with markers of neuroinflammation. As the primary resident immune cells in the brain, microglial cells would be the first-line candidate mediators of abnormal immune-brain dialogue. Accumulating evidence suggests that glial pathology is a prominent feature of depression, and that microglia alterations participate in the neuropathology of depression (Jo et al., 2015). Microglial cells are proficient phagocytes and regarded as CNS-resident immune cells (Prinz and Priller, 2014). They are the primary sentinels patrolling the CNS, alert to any potential harmful event including pathogen invasion (Ransohoff and Perry, 2009). Moreover, their role is also extended to debris clearance, trophic support to neurons, and synaptic pruning during neurogenesis (Blank and Prinz, 2013). Human post-mortem studies in

depressives who committed suicide and control subjects identified higher microglial activation in different brain regions, including the anterior cingulate cortex (ACC), the pre-frontal cortex (PFC) and the hippocampus, in the former group (Steiner et al., 2008; Torres-Platas et al., 2014). Moreover, the levels of the neurotoxic molecule quinolinic acid (QA), which is synthesized in microglia, were also found to be dysregulated in different brain regions: microglial QA expression was elevated in the cingulate cortex, and was decreased or unchanged in the hippocampus (Busse et al., 2015; Steiner et al., 2008). Shelton et al. (2011), using gene expression analysis, observed up-regulation of local inflammatory, apoptotic, and oxidative stress pathways in the PFC from depressed subjects (Shelton et al., 2011). Interestingly, increased binding of translocator protein (TSPO), a marker of neuroinflammation mainly expressed by microglia cells, has been observed in vivo in the PFC, ACC, and insula of currently depressed patients using PET imaging (Setiawan et al., 2015).

Another indication that inflammatory processes are intimately related to depression is the observation of increased prevalence of depression comorbid with a number of conditions (e.g. aging and obesity) and diseases (e.g. atherosclerosis, multiple sclerosis and rheumatoid arthritis), all of which are characterized by a chronic inflammatory component (Halaris, 2013; Maes, 2011).

The findings of increased peripheral and central inflammatory markers in depression cannot answer the question of whether inflammation has a causal role in determining depressive state or is actually determined by depression itself. Interestingly, administration of inflammatory cytokines or cytokine inducers can promote depressive-like behaviours in both laboratory animals and humans (Haroon et al., 2012). These data support the notion that inflammation may have a role in the aetiopathophysiology of depression.

3.2 Stimulation of the immune system impacts on sickness and depression-relevant behaviours

Activation of peripheral inflammation and the systemic release of inflammatory cytokines can exert profound effects on brain and behaviour as a result of communication between the periphery and brain (Haroon et al., 2012). It is known that infectious diseases are associated with profound behavioural disturbances. These are collectively termed sickness behaviour and include malaise, fatigue, depressed mood, anorexia, sleep disturbances, decreased physical and social activities, and cognitive impairments (Dantzer et al., 2008). Sickness behaviour can be interpreted as an adaptive response of the host's homoeostatic and behavioural priorities to facilitate an immune response and cope better with an infection, rather than simply a detrimental consequence of infection per se (Brydon et al., 2008; Hart, 1988). In experimental settings, administration of endotoxin (lipopolysaccharide, LPS) to humans induces a number of behavioural changes, including depressed mood, fatigue, and cognitive dysfunction (Hannestad et al., 2011b; Reichenberg et al., 2001). Several studies have investigated the behavioural effects of peripheral administration of the inflammatory cytokine interferon-α (IFN-α), a common treatment for infectious diseases including chronic hepatitis C (Raison et al., 2009). Chronic administration of IFN-α induces high rates of behavioural disturbance, including depression, in 30%-50% of patients treated with IFN-α (Schaefer et al., 2002). Compared to medically healthy patients with depression, patients with IFN-α-induced depression show increased psychomotor retardation and weight loss and less severe feelings of guilt (Capuron et al., 2009). Interestingly, pre-administration of the antidepressant paroxetine is an effective strategy for minimizing depressive symptoms induced by IFN-α therapy (Musselman et al., 2001). Experimental endotoxemia (i.e. LPS administration) in healthy humans decreases mood, increases prefrontal activation during the presentation of emotionally aversive stimuli (Kullmann et al., 2013), and enhances activity within the ACC during emotional face processing (Harrison et al., 2009), suggesting

a common pathophysiological basis for depression and sickness-associated mood change (Harrison et al., 2009).

Using preclinical models of inflammation-induced behavioural alterations, it has been shown that depression-relevant behaviours occur against a background of sickness behaviours (Moreau et al., 2008; Walker et al., 2013). Crucial points include whether or not the mechanisms underlying post-sickness depression-relevant behaviours are the same as or different from those underlying sickness, and whether or not they are the same as or different from those underlying stress-induced depression-relevant behaviour (Walker et al., 2013). For example, in mice, LPS-induced peripheral inflammation reduces incentive motivation in an operant test for reward by affecting willingness to exert effort for reward and not by reducing consumption of freely-available reward (Vichaya et al., 2014). Another relevant example is the effects of inflammation versus psychosocial stress on processing of aversive stimuli: peripheral administration of cytokine inducers such as LPS (Pugh et al., 1998) or CD40 agonist antibody (Cathomas et al., 2015a) decrease Pavlovian fear conditioning, whereas psychosocial stress increases Pavlovian fear conditioning (Azzinnari et al., 2014; Yu et al., 2011).

One shared candidate for the initiation of aetiopathophysiological pathways for sickness behaviour and depression is the increased brain levels of the pro-inflammatory cytokines IL-1β and TNF-α which follow peripheral immune system activation (for review see Dantzer et al., 2008). A second candidate pathway is altered metabolism of tryptophan (TRP) (Dantzer et al., 2008; Schwarcz et al., 2012). The pro-inflammatory cytokines IFN-γ, TNF-α, IL-1, and IL-6 induce expression of indoleamine 2,3-dioxygenase (IDO) (Campbell et al., 2014; Lestage et al., 2002), an enzyme expressed in various immune cells throughout the body, including dendritic cells, peripheral macrophages and microglia. IDO metabolizes TRP into kynurenine (KYN) along the KYN pathway (Haroon et al., 2012; Schwarcz et al., 2012). A second rate-limiting enzyme in the KYN pathway, also responsible for TRP-KYN conversion, is tryptophan-2,3-dioxygenase (TDO). TDO is mainly induced by corticosteroids and glucagon (Campbell et al., 2014), and inflammatory stimuli (Urata et al., 2014; Walker et al., 2013). In the periphery and brain, KYN catabolism results in increased levels of 3-OH-kynurenine (3-HK), quinolinic acid (QA) and/or kynurenic acid (KYNA) (Campbell et al., 2014). Tryptophan, KYN and 3-HK are readily transported across the blood-brain-barrier (BBB), while QA has to be produced in the brain from microglia cells (Schwarcz et al., 2012). In mice, IDO brain expression is increased at 24–48 h after LPS administration and correlates with LPS-induced behavioural effects (O'Connor et al., 2009). KYN pathway activation can result in excitotoxicity and oxidative stress in various brain regions and cell types, mediated by and impacting on various neurotransmitter systems, including glutamate, serotonin and dopamine (Cathomas et al., 2015a; Felger and Miller, 2012; Haroon et al., 2012). Effects of inflammation and of KYN pathway activation on DA system will be discussed in section 3.4.

3.3 The effects of stress on inflammation

As presented in section 1.3, chronic psychosocial stressors are key proximal risk factors for depression (Kendler et al., 1999; Slavich et al., 2009). Thus, data showing that acute and chronic stressors can activate an immune response represent a fundamental step linking stress and depression as it relates to the immune system (Haroon et al., 2012).

One of the earliest findings demonstrating that social stress can activate the immune system, obtained in humans, was that exposure to the Trier Social Stress Test (TSST), a public speaking and

mental arithmetic stressor, is associated with a rapid activation of NF-kB signaling in peripheral blood mononuclear cells (PBMCs) (Bierhaus et al., 2003). Interestingly, depressed subjects with an early life stress history exhibit an enhanced inflammatory responsiveness to the TSST, as indicated by higher IL-6 levels and NF-kB activation compared to healthy controls (Pace et al., 2006). Cohen et al. (2012) showed that stressful life experiences associated with long-term threat are associated with proinflammatory leukocytic phenotypes that are unresponsive to the anti-inflammatory actions of glucocorticoids (GCs). In accordance with these findings, chronic social stress in humans is associated with an increased representation of the immature proinflammatory monocyte transcriptome in PBMCs (Powell et al., 2013), and with functional resistance to glucocorticoids in monocytes, which enables activation of pro-inflammatory transcription control pathways (Miller et al., 2008a).

Preclinical research has investigated the effects of stress on the immune system, both in the periphery and in the CNS. One very well described murine stress paradigm is repeated social defeat (RSD) (for review see Reader et al., 2015). RSD causes immune changes both in the periphery (i.e. bone marrow, circulation, spleen) and in the brain. RSD induces splenomegaly that correlates with increasing accumulation of splenic $CD11b^+$ monocytes and granulocytes (Wohleb et al., 2012). Furthermore, social defeat induces GC resistance in splenocytes (Avitsur et al., 2002; Engler et al., 2008). Accumulation of neutrophils and monocytes in the circulation and in the spleen is associated with increased cell mobilization and myelopoiesis in the bone marrow (Engler et al., 2004). RSD stimulates the release into the circulation of the pro-inflammatory cytokine IL-6 (Wohleb et al., 2011). Regarding the effects of RSD on neuroinflammation, RSD leads to region-specific microglial activation and to increased inflammatory profile of microglia (Wohleb et al., 2011). Moreover, RSD increases monocyte/macrophage trafficking to the brain (Wohleb et al., 2013). These effects of region-specific microglial activation and monocyte trafficking associated with chronic stress have been observed in other paradigms of repeated stress (for review: Reader et al., 2015; Walker et al., 2014), including restraint stress (Hinwood et al., 2012; Hinwood et al., 2013; Kopp et al., 2013), footshocks (Ataka et al., 2013), CUMS (Couch et al., 2013; Pan et al., 2014). Besides being region-specific, microglia response to stress is also time-dependent. Kreisel et al. (2014) show a dynamic microglia response to CUMS in mice: hippocampal microglia cells were increased after 2 days of stress, but there was no change or even a reduction of microglia cells after 4 days or at the end of the 4-week stress protocol. Likewise, restraint stress increases the number of microglia only at day 4 of a 6-days stress protocol (Nair and Bonneau, 2006). Our laboratory has recently shown that CSD in mice leads to increased levels of the pro-inflammatory cytokines IL-6 and TNF-α, splenomegaly, and brain region-specific dysregulation of the inflammatory transcriptional signature (Azzinnari et al., 2014). Specifically, in the ventral hippocampus (HIPP), for 11 of the 54 genes with altered expression in CSD mice, the pro-inflammatory cytokines TNF, IL-6 and IL-3 were major upstream regulators of expression. The up-regulated genes included the TNF-receptor superfamily gene *Tnfrsf25* (Azzinnari et al., 2014). In AMYG, CSD led to dysregulation of genes related to canonical pathways of "T cell receptor signalling" and "CCR5 signalling in macrophages", suggesting that CSD initiated changes in immune-inflammation transcription processes in this brain area (Azzinnari et al., 2014). In mPFC, de-regulated canonical pathways were immune-inflammatory, namely "IL-12 signalling and production of macrophages" and "production of nitric oxide and reactive oxygen species in macrophages" (Azzinnari et al., 2014).

As presented in section 3.2, the KYN pathway can be activated by inflammatory signaling and it regulates immune system-mediated behavioural changes (Dantzer et al., 2008; Schwarcz et al.,

2012), possibly via its action on neurotransmitters systems (Felger and Miller, 2012). Interestingly, stress-induced inflammation has also been found to activate the KYN pathway. CUMS in mice increases plasma KYN levels and brain 3-HK content (Agudelo et al., 2014), while restraint stress increases TDO mRNA expression and enzymatic activity in the liver (Gibney et al., 2014). Chronic stress in mice up-regulates *Ido* mRNA expression in the dorsal raphe (Couch et al., 2013). Our lab has recently shown CSD activation of the kynurenine pathway in periphery and brain regions: in blood, KYN and 3-HK were increased in CSD versus CON mice. Co-occurring with these increased plasma levels of TRP metabolites, CSD mice had increased brain levels of KYN and 3-HK in AMYG and HIPP. Interestingly, CSD-induced KYN pathway activation and increased conditioned fear were reversed by pharmacological KYN pathway antagonism (i.e. IDO inhibitor) (Fuertig et al., in prep).

In terms of the mechanisms by which stress activates the inflammatory response, attention has been focused on the sympathetic nervous system (SNS) (Haroon et al., 2012), with the hypothesis that stress-induced increased sympathetic tone leads to the release of pro-inflammatory cytokines in the periphery (Elenkov et al., 2000; Powell et al., 2013; Rasouli et al., 2011). Indeed, catecholamines acting through α- and β-adrenergic receptors have been shown to increase cytokine expression in both the brain and the periphery of rats exposed to uncontrollable stressors (Johnson et al., 2005). More specifically, Powell et al. (2013) have shown that social stressors up-regulate bone marrow production of $Ly6C^{hi}$ proinflammatory monocytes and that these effects are mediated by β-adrenergic receptors. Pharmacological β-adrenergic antagonism (i.e. propranolol) has been shown to reduce stress-induced inflammation (Johnson et al., 2005), up-regulation of bone marrow myelopoiesis (Powell et al., 2013), microglia activation, and behavioural changes (Wohleb et al., 2011).

3.4 The DA system: a converging node for stress-induced inflammation

Several hypotheses have been proposed to account for the association of depression with increased inflammatory biomarkers and the capacity of (stress-induced) inflammatory stimuli to induce depressive symptoms (Haroon et al., 2012). These include the effects of stress and inflammation on neural plasticity, neuroendocrine function, and neurotransmitter metabolism (Haroon et al., 2012). The latter mechanism has received considerable attention in numerous human and rodent studies. Several neurotransmitter systems are affected by inflammation, including serotonin (Maes et al., 2012; Miller et al., 2013), dopamine (de Pablos et al., 2014; Miller et al., 2013), and glutamate (Dantzer and Walker, 2014; Steiner et al., 2012). In the psycho-neuro-immune hypothesis for depression presented here it is hypothesized that stress-induced inflammation targets basal ganglia and DA pathways, thus influencing the emergence of depression symptoms (Felger and Miller, 2012).

Various lines of experimental evidence indicate that inflammation can affect DA signaling. In rat, peripheral administration of LPS increases extracellular levels of DA metabolites (DOPAC and HVA) in the NAcc via increased DAT activity (van Heesch et al., 2014). Peripheral TNF-α can also increase HVA NAcc levels (van Heesch et al., 2013). Moreover, high doses of peripheral LPS lead to a decrease in tyrosine hydroxylase (TH)-expressing neurons in the SN (Qin et al., 2007); an effect that is partially mediated by LPS-induced microglia activation and the subsequent induction of oxidative stress in DA cells (Gao et al., 2002; Qin et al., 2013). In Parkinson's disease, oxidative stress-mediated death of DA neurons has been proposed as a mechanism for DA cell degeneration (Kannarkat et al., 2013; Miller et al., 2009b). Human studies with endotoxin challenge have shown greater increases in self-reported and observer-rated depressed mood over time, as well as significant reductions in ventral striatum activity to monetary reward cues (Eisenberger et al., 2010). Moreover, peripheral inflammation

following typhoid vaccination is associated with altered SN activity and psychomotor slowing (Brydon et al., 2008), and with mood deterioration and reduced sACC-NAcc functional connectivity during emotional stimuli processing (Harrison et al., 2009).

Rodent studies have investigated the link between inflammation and DA function using a model for multiple sclerosis, i.e. experimental autoimmune encephalomyelitis (EAE). It has been shown that EAE-mediated neuroinflammation impairs striatal DA neurotransmission, as indicated by reduced DA metabolism (i.e. reduced DA turnover) (Balkowiec-Iskra et al., 2007) and reduced DA release (Gentile et al., 2015). Given the high comorbidity of depression with multiple sclerosis (Maes, 2011), findings of EAE-induced DA dysfunction might be relevant for the identification of shared (inflammatory) pathophysiological routes (Feinstein et al., 2014; Gold and Irwin, 2006).

As presented in section 3.2, IFN-α therapy induces depression-relevant behaviours in humans and laboratory animals. These behavioural effects co-occur with mesolimbic DA dysfunction. Capuron et al. (2007) showed basal ganglia hyper-metabolism and symptoms of fatigue during IFN-α therapy. Increased basal ganglia glucose metabolism has been demonstrated in PD patients and might reflect the degeneration of inhibitory circuits, consequent to the loss of DA cells in the SNpc (Capuron et al., 2007; Wichmann and DeLong, 2003).

Given the effects of inflammatory signaling on DA transmission, it can also be hypothesized that stress-induced immune system activation might exert detrimental effects on the DA system. de Pablos et al. (2014) showed that in rats chronic stress exacerbates microglial activation after injection of a pro-inflammatory stimulus such as LPS in the ventral mesencephalon, leading to an increase in the death of DAergic neurons in the SN. Moreover, psychosocial stress (CSD) in mice activates microglia, increases COX-1 microglia expression in the VTA, and COX-1-dependent prostaglandin E2 (PGE2) increase attenuates mesocortical DA signaling (Tanaka et al., 2012).

Interestingly, Savitz et al. (2015a) have shown that in depressed subjects the KYN pathway is activated and that the KYN/TRP ratio is inversely associated with striatal volumes in the depression sample, suggesting that activation of the KYN pathway might affect striatal volume in depressed patients to an extent that has clinical significance (Savitz et al., 2015a).

4. Aims of the thesis

Aims of Study A: Chronic depletion of nucleus accumbens dopamine in mice leads to impaired reward and aversion processing relevant to motivation pathologies

There is evidence that decreased DA neurotransmission in the mesocorticolimbic pathways leads to impaired reward and aversion processing and could underlie major psychopathologies, including motivational impairments that are common in depression. 6-OHDA NAcc injection is a well-established method to achieve chronic VTA-NAcc DA depletion and investigate its effects on behaviour. Loss-of-function studies have been conducted primarily with rat and reward processing. Equivalent mouse studies are rare, and the aim of Study A was to investigate the effects of 6-OHDA-induced chronic NAcc DA depletion on mouse behaviour using the following test battery: reward motivation in a progressive ratio schedule (PRS) operant test, approach-avoid responding in a learned non-reward (LNR) operant test, footshock escape behaviour in a learned helplessness (LH) operant test, footshock avoid-escape behaviour in a treadmill operant test, and freezing behaviour in a Pavlovian fear conditioning test. A further rationale of Study A was to facilitate understanding of DA contribution to the disrupted behaviours induced by environmental stress in mice; for example, chronic social defeat (CSD) impairs animals' reward seeking and active attempts to control aversive stimuli, and impacts on VTA-NAcc DA function (see Study B and C). Therefore, each mouse behavioural test included in Study A is relevant to dimensions of reward or aversion processing often disrupted in psychiatric disorders, and studied also in Study B or C.

Aims of Study B: Mouse chronic social stress induces peripheral-CNS inflammation, dopamine deregulation and disrupted processing of rewarding stimuli

Depression is often preceded by stressful life events and correlates with high levels of inflammatory markers, at least in a subpopulation of patients. Moreover, experimental findings suggest the involvement of DAergic dysfunction in depressive symptoms. The psycho-neuro-immune hypothesis for depression postulates that the inhibitory effects of stress-induced inflammatory signaling on DA neurotransmission represent a link between exposure to stressful environments and the emergence of depression-relevant behavioural abnormalities. Thus, the first aim of Study B was to investigate the effects of CSD on reward motivation using consummatory and appetitive-operant tests and on cognitive flexibility in the face of changing response-reward contingency using a learned non-reward (LNR) test. Secondly, we aimed to extend the understanding of the inflammatory response that is elicited by CSD, characterizing the activation of the innate and adaptive immune system and of the kynurenine pathway, with focus on the mesolimbic DA system. Lastly, we assessed the effects of CSD on the mesolimbic DA system, with a focus on DA signaling in the mesocorticolimbic pathway.

Aims of Study C: Mouse chronic social stress disrupts reward motivation and cognitive flexibility and certain of these effects are responsive to the antidepressant agomelatine

Depression is characterized by reduced motivation for reward and impaired reward learning, while responsiveness to reward lies in the normal range of functioning. In humans, these behavioural domains have been tested using the effort expenditure for rewards task (EEfRT), the probabilistic reversal learning (PRL) and the sweet taste test, respectively. In Study C, the effects of CSD on reward-directed motivation and reward learning were assessed using a progressive ratio schedule (PRS) test and an Intellicage operant sucrose test, and the PRL test, respectively. The second major aim of Study C was to investigate the effects of the newly developed antidepressant agomelatine (Valdoxan), particularly in terms of its efficacy in reversing CSD-induced deficits in reward processing.

Study A

Depletion of nucleus accumbens dopamine leads to impaired reward and aversion processing in mice: relevance to motivation pathologies

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Abstract

Experimental reduction of dopamine (DA) neurotransmission in the mesocorticolimbic pathways leads to impaired reward and aversion processing, and could underlie the motivational impairments that are common in depression and schizophrenia. The ventral tegmental area-nucleus accumbens (VTA-NAcc) DA projection is fundamental to these effects. Injection of the neurotoxin 6-hydroxydopamine (6-OHDA) in the NAcc is a well-established method for chronic NAcc DA depletion. Whilst stress-model and molecular-genetic studies of DA function focus on mice, 6-OHDA NAcc studies have been conducted primarily with rat. The aim of the current study was to investigate effects of 6-OHDA-induced NAcc DA depletion on C57BL/6 mouse behaviour using the following test battery: reward motivation in a progressive ratio schedule (PRS) operant test, approach-avoidance responding in a learned non-reward (LNR) operant test, footshock escape behaviour in a learned helplessness (LH) operant test, footshock avoid-escape behaviour in a treadmill operant test, and freezing behaviour in a Pavlovian fear conditioning test. Relative to vehicle controls, NAcc DA-depleted mice exhibited reduced responding: (i) for gustatory reward under effortful conditions in the PRS test; (ii) to a stimulus recently associated with gustatory non-reward in the LNR test; (iii) to footshock recently experienced as uncontrollable in the LH test; and (iv) to footshock that could only be avoided-escaped by physical effort in the treadmill test. Taken together, these findings add significantly to the comparative evidence that NAcc DA is a major regulator of motivational processing of both reward and aversion. They indicate the need for translational study of the targetting of pathophysiological DA function for the treatment of motivational psychopathologies in various psychiatric disorders, and the suitability of mouse, in addition to rat, models for this research.

Introduction

Dopamine (DA) neurons residing in the ventral tegmental area (VTA) innervate several brain regions, and primary among these are the hippocampus, amygdala, prefrontal cortex (PFC) and nucleus accumbens (NAcc) (Lammel et al., 2008; Sesack and Grace, 2010). The NAcc is part of the ventral striatum and has extensive connectivity with other brain regions: it receives excitatory inputs from the PFC and the amygdala, and inhibitory afferents from the ventral pallidum (VP); major projection regions of the NAcc include the VP and, reciprocally, the VTA (Sesack and Grace, 2010). Nucleus accumbens DA signalling modulates behavioural responsiveness to stimuli, in terms of stimulus-stimulus learning, response-outcome learning, and the motivation underlying behaviour directed at positive stimuli and negative stimuli (Salamone and Correa, 2012; Wise, 2004). The motivation functions of NAcc DA is the focus of the present study, and a major rationale for the study is that pathologies in motivation towards positive-valence and negative-valence stimuli are characteristic of various psychiatric disorders including depression and schizophrenia (Eshel and Roiser, 2010; Wang et al., 2015). Reduced DA function has been hypothesized as a major pathophysiological factor in depression (Dunlop and Nemeroff, 2007) and negative symptomatology in schizophrenia (Whitton et al., 2015). There is evidence for altered DA neurotransmission in each of these disorders (Bowden et al., 1997; Mitani et al., 2006; Rice et al., 2014).

Much of the definitive evidence for the importance of NAcc-DA function in the modulation of goal-directed behaviour has been obtained in loss-of-function experiments conducted in rats. Infusion of the monoamine neurotoxin 6-hydroxydopamine (6-OHDA) into the NAcc, leading to specific degeneration of DA fibre terminals (Stott and Barker, 2014), is the most well-established method (Aberman and Salamone, 1999), and has primarily been applied to study the importance of NAcc DA in the regulation of gustatory stimulus processing. In rat, in free-choice situations, NAcc DA depletion delays development of preference for sucrose solution relative to water (Martinez-Hernandez et al., 2012), NAcc DA depletion impairs both the operant acquisition of response-outcome learning and the motivation to respond, with the latter effect being most pronounced under conditions of high effort (Aberman and Salamone, 1999). Such findings demonstrate that NAcc DA regulates the motivation for - the “wanting” of - sweet-tasting stimuli (Nunes et al., 2013b). In contrast, NAcc DA does not regulate appetite for homeostatic dietary need (Cousins and Salamone, 1994), or hedonic responding to - the “liking” of - sweet stimuli (Smith et al., 2011). In addition to the evidence that NAcc DA regulates reward learning and motivation, there is interest in the involvement of DA in regulation of the behavioural flexibility required under conditions of changing response-outcome contingency. For example, VTA DA projections to the prefrontal cortex (PFC) have been proposed to actively maintain goal-directed behaviour in the face of distraction (Cools et al., 2011; van Schouwenburg et al., 2010). Reversal learning involves inhibition of a previously reinforced response i.e. inhibition of perseveration, and activation of a previously non-reinforced response i.e. suppression of learned irrelevance/learned non-reward. Serotonin within the PFC has been demonstrated to suppress perseveration, in common marmoset (Clarke, 2004), and glutamate within the orbital PFC has been demonstrated to suppress learned irrelevance, using a reversal-learning test that separated perseveration and learned non-reward, in rat (Tait and Brown, 2007). Recently, a mouse test for learned non-reward (LNR) was described (Nilsson et al., 2012), and can be used to study the involvement of NAcc DA or PFC DA in the regulation of this form of behavioural flexibility. The first aims of the present study were to investigate the effects of 6-OHDA depletion of NAcc DA in mice on their motivation for sucrose-reinforcer using a progressive ratio schedule test; and of 6-OHDA depletion of NAcc DA or medial PFC (mPFC) DA on their flexibility in the face of changing response-outcome contingency using the LNR test.

Nucleus accumbens DA also modulates goal-directed behaviour relative to negative stimuli. Dopamine is released acutely in the NAcc in response to unconditioned aversive stimuli e.g. electric footshock (Young et al., 1993) and social stress (Cabib and Puglisi-Allegra, 2012). In the case of inescapable/uncontrollable stimuli, the initial increase in NAcc DA is followed by a decrease below pre-stress tonic levels (Cabib and Puglisi-Allegra, 2012). As with positive stimuli, NAcc DA appears to modulate learning and motivational aspects of behaviour directed at aversive stimuli. In rat, depletion of NAcc DA leads to a deficit in operant lever pressing or two-way shuttling, to avoid-escape footshock (McCullough et al., 1993; Wenzel et al., 2015). In DA-deficient mice in which DA expression could be reactivated region-specifically, restoration of DA signalling to both entire striatum and amygdala was necessary and sufficient to enable mice to learn two-way active avoidance (Darvas et al., 2011). Also in mice, DA depletion in VTA and substantia nigra pars compacta led to increased sensitivity to inescapable footshocks expressed as a subsequent response deficit in a lever-press escape test i.e. learned helplessness (Winter et al., 2007). With respect to aversive stimulus-stimulus learning, studied primarily using Pavlovian fear conditioning, conditioned stimuli (CS), e.g. tone, that predict footshock, increase DA release in the NAcc (Pezze and Feldon, 2004; Wenzel et al., 2015). Whether NAcc DA depletion affects CS fear conditioning or expression remains to be investigated (Pezze and Feldon, 2004). To increase understanding of the involvement of NAcc DA in aversive stimulus processing, particularly in terms of motivation to respond adaptively to them, the second aims of the present study were to investigate the effects of 6-OHDA depletion of NAcc DA in mice on their active escape of footshock in a learned helplessness (LH) test (Pryce et al., 2012), and their active avoidance-escape of footshock in a treadmill running test (Azzinnari et al., 2014). Given that the mPFC is critical to the processing of stimulus un/controllability in the rat (Amat et al., 2008), the effects of mPFC DA depletion were also studied in the LH test. The third aim was to study effects of NAcc DA depletion in the Pavlovian fear conditioning test.

Animal models are essential to understanding the contributions of NAcc DA and mPFC DA to adaptive and disrupted motivational processing of rewarding and aversive stimuli. Reduced motivation to engage with reward underlies the core depression symptom of loss of interest and core negative symptoms in schizophrenia, and is prominent in the mental health research domain criterion (RDoC), Positive valence systems (Cuthbert and Insel, 2013). Altered reactivity to aversive stimuli is a major construct in the RDoC, Negative valence (Cuthbert and Insel, 2013). Altered reactivity to and motivation to cope with aversive stimuli could well contribute to the core depression symptoms of low mood and high fatigue (Eshel and Roiser, 2010). For example, perceived absence of control of aversive stimuli can lead to helplessness, including reduced motivation to attempt control (Nunes et al., 2013b; Pryce et al., 2011a; Treadway and Zald, 2011), and increased emotional reactivity to and decreased motivation to control aversive stimuli both contribute to high fatigue (Demyttenaere et al., 2005). As described above, it is rat studies that have contributed most to current understanding of mesolimbic DA regulation of motivation and cognition relative to positive and negative stimuli. Currently, the 6-OHDA DA depletion approach has rarely been applied in mouse, but there is nonetheless increasing emphasis on applying environmental manipulations and molecular-genetic tools in mice to study DA function and dysfunction. For example, chronic social defeat stress in mice has been shown to impair reward-directed behaviour (Chaudhury et al., 2013; Lammel et al., 2014) and active attempts to control aversive stimuli (Azzinnari et al., 2014), and optogenetic methodology has been applied to stimulate VTA DA neurons and reverse some of these effects (Chaudhury et al., 2013; Lammel et al., 2014; Tanaka et al., 2012). We aimed to investigate whether 6-OHDA NAcc, and in some tests mPFC, DA depletion impacted on mouse reward and

aversion processing in a similar manner to that described for rat, and were particularly interested in its motivational effects, given the importance of motivation pathologies in psychiatry.

Materials and methods

Animals

The study was conducted with male C57BL/6J mice (Janvier Labs, Saint-Berthevin, France) aged 10 weeks and weighting 26.1 ± 2.0 g at the time of surgery. Mice were maintained in pairs in type 2L cages and on a reversed 12:12 h light-dark cycle (lights off at 07:00 h) in an individually-ventilated caging system, with temperature at 20-22°C and humidity at 50-60%. The standard diet was complete pellet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water, both available continuously and *ad libitum* unless stated otherwise below for experimental reasons. All procedures were conducted under a permit for animal experimentation (No. 170/2012) issued by the Veterinary Office, Zurich, Switzerland, in accordance with the Animal Protection Act (1978) Switzerland.

Injection of 6-hydroxydopamine (6-OHDA)

At 30 min prior to injection of 6-OHDA or vehicle, mice received i.p. injections of desipramine hydrochloride (Sigma-Aldrich, 50 and 35 mg/kg for NAcc and mPFC lesion, respectively) and escitalopram oxalate (H. Lundbeck A/S, Valby, Denmark, 5 mg/kg), in order to block 6-OHDA uptake by noradrenaline and serotonin neurons, respectively. Mice were anaesthetised with a cocktail of fentanyl 0.04 mg/ kg, midazolam 4 mg/kg and medetomidine 0.4 mg/kg, i.p.. Using a stereotaxic frame (WPI, Berlin, Germany) and 10 µl syringe (NanoFil, WPI) equipped with a 33G needle (WPI), mice received bilateral injections of 6-OHDA hydrochloride (Sigma-Aldrich) or vehicle in the NAcc or mPFC. 6-hydroxydopamine was dissolved in 0.9% saline containing 0.02% ascorbic acid (AA). Coordinates, established using a mouse brain atlas (Franklin and Paxinos, 2008) and pilot injections of methylene blue, were, relative to bregma, for NAcc, AP +2.0, ML ±0.7, DV -4.6 mm, and for mPFC, AP +2.4, ML ±0.3, DV -2.4 mm. Injection parameters for 6-OHDA were, for NAcc, 1.5 µg or 2 µg/0.5 µl/2.5 min, and for mPFC, 1.5 µg/0.2 µL/2 min, and the same volume and rate were used for vehicle injections (Veh: 0.02 % AA in saline). The needle was left in place for a further 3 min to allow tissue diffusion, before being retracted slowly. A period of 10-12 days, sufficient for mouse post-surgery recovery and for degeneration of DA fibres (Stott and Barker, 2014), was allowed between injection and behavioural testing. Body weight was measured on days 1-10 post-surgery.

Behavioural testing

Operant tests using gustatory positive reinforcement

Apparatus. For the progressive ratio schedule (PRS) and learned non-reward (LNR) experiments, training and testing were conducted using operant boxes (TSE Systems GmbH, Bad Homburg, Germany), details of which are given elsewhere (Ineichen et al., 2012). Briefly, nose-poke ports detected mouse nose poke responses via an infra-red beam, and port position and number changed according to the test (PRS or LNR). Sugar pellets (14 mg, Dustless Precision Pellets, TSE Systems GmbH) were delivered singly into the feeder port, which was situated adjacent to the single nose-poke port in the PRS test and in the wall opposite the two nose-poke ports in the LNR test. Pellet delivery was signalled by a tone from a speaker. Pellet retrieval was detected via infra-red beam.

Restricted feeding protocol. For 5 days, mouse daily body weight and daily food intake were measured to obtain mean baseline values. Beginning one week before and continuing during the

operant training phases of the PRS and LNR experiments, mice were food restricted and kept at 90% of their free-feeding (baseline) body weight, to ensure motivation for operant training.

Progressive ratio schedule (PRS) test

Naive mice (N=24) were initially trained to nosepoke into a single port to obtain a sucrose pellet under a fixed ratio 1 (FR1) schedule. Mice required 8-12 sessions to acquire the defined criterion for operant learning, which was 2 consecutive sessions at which ≥ 30 pellets were earned at FR1. For PRS testing, mice were returned to 100 % baseline body weight; this was to reduce their appetite and thereby to increase test sensitivity to detecting motivation for the sweet taste of sucrose pellets rather than their calorific content. Mice were then given 3 sessions of PRS testing and the mean score of the last two sessions was used to counter-balance subject allocation to experimental (NAcc Veh vs 6-OHDA) groups. The PRS test parameters were: start ratio (required number of nose-pokes on first trial) = 1, repetition factor (number of consecutive trials for which the ratio remained constant) = 5, and step-wise linear fixed ratio increase (number of nose-pokes by which the ratio increased per increment) = 3, e.g. reward ratio on trials 1-5 = 1 response, on trials 6-10 = 4 responses, on trials 11-15 = 7 responses, and on trial 16-20 = 10 responses, and so on. The break point, i.e. minimum time without at least 1 response that signalled end of the session, was 600 s, and maximum session duration was 40 min. After surgery + 10-12 days, mice, weighing 100 % baseline body weight (104 $\pm$ 3 %) were given the PRS test. The measures of interest were: cumulative number of pellets earned and ratio attained at 10, 20, 30 and 40 min after test onset, overall mean time between collection of pellet *n* and the first operant response for pellet n+1 (post-reinforcement pause, PRP), and absolute peak response rate (PRR) calculated as the highest overall response rate within a ratio and expressed as responses/min (Bezzina et al., 2008).

Learned non-reward (LNR) test

The LNR test was based on that described by Nilsson et al. (2012). Naive mice (N=19 in NAcc experiment, N=25 in mPFC experiment) were kept at 90-95% baseline body weight throughout LNR testing. Details of operant training for this test are given in *Supplementary Information*. Briefly, mice were trained to initiate each trial by one operant nosepoke into the feeder port, which illuminated a stimulus nose-poke port in the opposite, stimulus wall; one response in the stimulus port activated signalled delivery of a sucrose pellet into the feeder port, which the mouse returned to and retrieved the pellet. The criterion for completion of training was 2 consecutive sessions at which ≥ 49 pellets were earned. There were three spatial locations (left, middle, right) in the stimulus wall. The first stage of the test was a two-choice spatial discrimination (SD): following trial initiation, the subject had to choose between two stimulus nose-poke ports whilst a blank panel occupied the third location. A response in the correct stimulus port led to delivery of a sucrose pellet in the feeder port and an incorrect response led to a 5 s timeout. Mice were allowed 10 s to initiate a trial and 6 s to make a choice response. The SD stage consisted of 70 trials divided into seven 10-trial blocks; SD learning criterion was nine correct responses within any 10-trial block, and if the subject did not attain criterion the SD stage was repeated on the next day. On the day after attaining SD criterion, the second stage, learned non-reward (LNR), was presented: the incorrect stimulus port location at SD became the correct port, the previously correct stimulus port was removed and replaced by the blank panel, and the blank panel was replaced by a stimulus port and this became the new location for the incorrect port. Number of trials, learning criterion, and times allowed for trial initiation and choice responding, were all identical to those at the SD stage. At the LNR stage, the subject must

overcome nonreinforcement-avoid behaviour and acquire reinforcement-approach behaviour at the same stimulus port, in the absence of the possibility to perseverate in responding at the previously correct port (Nilsson et al., 2012). Measures of interest for SD and LNR were: omissions to initiate a trial (OIT), omissions to make a choice response (OR), incorrect choice response (IR), the sum of OIT, OR and IR, referred to as total errors (TE), and correct choice responses (CR). Prior to 6-OHDA infusion, mice were tested on three stages of SD+LNR so that the effects of DA depletion could be assessed against a stable baseline of SD and LNR behaviour. Between the completion of LNR and the subsequent SD stage, mice were given one session with only the central stimulus port available. Total errors in the third LNR stage were used to counter-balance subject allocation to experimental (Veh vs 6-OHDA) groups. After surgery + 10-12 days, mice, weighing 90-95 % (92 $\pm$ 4 %, NAcc Expt; 93 $\pm$ 5 %, mPFC Expt) baseline body weight were tested on the fourth stage of SD+LNR.

Operant tests using footshock negative reinforcement

Learned helplessness (LH) test

The LH test was conducted using a fully-automated apparatus (Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany), details of which are given in (Pryce et al., 2012). Briefly, an arena was positioned on an electrifiable grid and contained a central divider with an opening ("gate") via which mice could transfer from one side of the arena to the other. The test was based on the evidence that pre-exposure of mice to inescapable (uncontrollable) footshocks in this context leads to a subsequent deficit in two-way escape behaviour to the same stimulus in the same context, relative to mice pre-exposed to the same amount of escapable (controllable) footshock and to mice naive of footshock pre-exposure i.e. a validated specific LH effect (Pryce et al., 2012). In the present experiment, only the inescapable condition was used, to assess whether NAcc or mPFC DA depletion affected responses to inescapable footshock, and whether it affected responses to the shift from inescapable to escapable footshock (whether DA depletion effects were specific to pre-exposure to inescapable stimuli or also altered responses to pre-exposure to escapable stimuli was not tested). Naive mice were allocated randomly to 6-OHDA and Veh groups, N=27 in the NAcc experiment and N=24 in the mPFC experiment. After surgery + 10-12 days, day 1, mice were placed in the arena for 15-min without footshock to habituate them to the two-way arena and assess their locomotor activity. On days 2-4 (Inescapable footshock exposure) mice were exposed to 24 inescapable footshock (IS) trials/day, at 0.15 mA; footshock duration was variable, with a mean of 3.5 s, 2.7 s and 3.3 s on days 2, 3 and 4, respectively (maximum per trial footshock duration was 5 s on each day), and using a variable inter-trial interval (ITI) of 50 ± 40 s. On day 5 (Escape test) mice were exposed to 30 escapable footshock (ES) trials at 0.20 mA and with constant maximum footshock duration of 5 s/trial. The following measures were calculated for the escape test: total escape failures, mean escape latency, mean locomotor activity during ITI (arbitrary units/min, a.u./min), mean % time spent freezing during ITI (freezing defined as zero a.u. moved for at least 2 s), and distance moved during footshock (footshock reactivity, a.u./s).

At day 1 specifically, 6-OHDA mice exhibited decreased locomotion (see Results). In order to investigate whether this was associated with increased anxiety in a novel environment, an additional experiment was conducted in which mice were first given a 15-min habituation session in the two-way arena, on the following day received NAcc 6-OHDA (N=10) or Veh (N=10) infusion, and then given further activity tests in the same arena at 14 and 15 days post-surgery.

Treadmill test

The same mice as those used in the study of NAcc DA depletion effects in the LH test were used for this experiment, with an interval of 2 days. The treadmill test requires the mouse to run on an inclined (5°) plane to avoid or escape an electrified grid; it has been used to demonstrate a deficit in running at fast speed specifically in mice exposed to chronic psychosocial stress (Azzinnari et al., 2014). The test is suitable to detect effects of manipulations on two states (although not concurrently): the absolute level of effort the subject exerts to avoid-escape punishment - if this level is exceeded by the treadmill speed the subject will receive the maximum footshock amount shortly after test onset; the cumulative level of effort the subject exerts to avoid-escape punishment - the maximum footshock amount will accumulate gradually and exponentially and the mouse will approach the maximum test duration. On experimental day 1 (training), mice were placed on the treadmill for 2 min at a speed of 0 cm/s, 5 min at 15-20 cm/s at 1 min increments, and 5 min at 20 cm/s. Total number and duration of footshocks were scored automatically; the cumulative maximum duration of footshock any mouse could receive was 20 s, and the training session was terminated if this was reached. On day 2, a pre-test session consisted of 2 min at 0 cm/s and 5 min at 20 cm/s. This was followed immediately by the test session, which consisted of 20 min at 23 cm/s (or less if cumulative total footshock duration reached 10 s). Test measures were running time at 23 cm/s (maximum 20 min) and total cumulative footshock duration (maximum 10 sec).

Pavlovian CS-footshock fear conditioning test

For this test of aversive stimulus-stimulus conditioning the same apparatus was used as for the LH test. Naive mice (N=19) were allocated randomly to NAcc 6-OHDA and Veh groups. After surgery + 10-12 days (day 1), mice were placed on the grid in the arena (without central gate) for 15 min without footshocks to assess their locomotor activity and habituate them to the two-way arena (context). On day 2 (Conditioning), mice were placed back in the arena and exposed to 6 trials of 20 s tone (5 kHz, 85 dB, conditioned stimulus, CS) the final 2 s of which were contiguous with an inescapable footshock of 0.20 mA (unconditioned stimulus, US), and with inter-trial intervals (ITI) of 120 s. On day 3 (Expression test), mice were returned to the arena for a 20-min context test (20 x 1-min bins), followed immediately by a CS test in the same context comprising 12 x 30 s CS separated by ITI of 90 s. The test measure was % time spent freezing during CS or ITI trials (Cathomas et al., 2015c).

Hot plate pain test

As an independent assessment of effects of NAcc DA depletion on pain sensitivity, a hot plate test was conducted in mice studied in the LH + Treadmill experiment and in mice studied in the fear conditioning experiment, with an inter-test interval of 1 day. The hot plate test was conducted using a programmable thermoelectric heating plate (Teca, Chicago IL, USA) set at 50°C (Pryce et al., 2012). The latency (s) until first occurrence of one of the following behaviours was scored: licking a forepaw, licking a hind paw, lifting a hind paw, jumping. The maximum test duration was 60 s.

Ex vivo determination of brain tissue monoamine levels

At 1-2 days after completion of a behavioural experiment, the fresh brain was collected, frozen on powdered dry-ice and stored at -80°C. Frozen brains were sectioned coronally at 1.0 mm intervals using a stainless-steel brain matrix (Plastics One, model MMCS-1, Roanoke VA, USA) (Azzinnari et al., 2014). The NAcc, mPFC, caudate-putamen (CPu) and amygdala (Amyg) were identified using a mouse brain atlas (Franklin and Paxinos, 2008) and micro-dissected bilaterally using a brain punch (∅ = 1-

mm, Stoelting Europe, Dublin, Ireland) (Azzinnari et al., 2014) and the two punches per region and mouse were processed together. Mean total tissue weights per region were: NAcc 1.7 mg, mPFC 1.3 mg, CPu 1.3 mg, Amyg 1.6 mg.

HPLC-ED for DA and 5-HT. For tissue homogenization, 100 µl (mPFC, Amyg), 250 µl (NAcc) or 300 µl (CPu) ice-cooled perchloric acid (0.4 M) was added to each sample. Ultrasonication was conducted for 5 s at 30% power (VibraCell, VCX130PB, Sonics and Materials, Inc., Newtown CT, USA), followed by centrifugation at 16,000×g for 10 min at 4°C (CT15RE, VWR/Hitamachi, Lutterworth, UK). The supernatant was passed through a 0.22 µm filter (Minisart RC4, Sartorius AG, Göttingen, Germany) and kept on ice until analysis. High performamce liquid chromatography (HPLC) and electrochemical detection (ED) were conducted for DA and 5-HT and their respective major metabolites, dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), according to Oeckl et al. (2012). Briefly, isocratic separation of DA and 5-HT was carried out with a reversed-phase C18 column (YMC-Pack ODS-AQ, 100×2.1 mm, S-3 µm, YMC Europe GmbH, Dinslaken, Germany). The mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium salt, 1.0 mM $Na_2EDTA \times 2H_2O$, 8.0 mM NaCl, 100 mM $NaH_2PO_4 \times 2H_2O$ (pH 3.80), mixed with 9.3% acetonitrile, and was delivered at a flow rate of 0.4 ml/min. For ED an electrochemical cell with a glassy carbon electrode and an ISAAC Ag/AgCl reference electrode (VT-03, Antec, Zoeterwoude, Netherlands) was used. Homogenates (20 µl) were injected onto the HPLC system using an autosampler (ASI-100T). Concentrations of monoamines and metabolites were calculated using an external standard calibration and expressed as ng/mg brain tissue.

Radioenzymatic assay for noradrenaline. Following tissue homogenization (see above) PFC, NAcc and Amyg samples were further diluted (1:1, 1:10 and 1:5, respectively) in artificial cerebrospinal fluid (aCSF). For NA quantification, a radioenzymatic assay was used as described previously.(Lu et al., 2008) This assay involves COMT-catalyzed O-methylation using [3H]S-adenosylmethionine as methyl donor and separation of the resulting [3H]normetanephrine by thin-layer chromatography (TLC).

Immunohistochemistry for tyrosine hydroxylase

In addition to quantification of DA tissue levels using HPLC-ED, 6-OHDA depletion of DA fibre terminals in NAcc (and surrounding regions) was also assessed qualitatively using tyrosine hydroxylase (TH) immunohistochemistry. In an additional cohort of mice, following NAcc 6-OHDA (N=3) or Veh (N=3) infusion + 12 days, mice were deeply anaesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) and fixative (4% paraformaldehyde (PFA) in PBS). Brains were post-fixed over-night in 4% PFA, and cryoprotected in 30% sucrose for 48h. Brains were frozen on powdered dry ice and cut at 40 µm with a microtome (Zeiss). Brain sections were collected in antifreeze solution and stored at -20°C until analysis. Free-floating sections were washed with PBS, quenched with 3% H_2O_2 for 15 min, blocked with normal goat serum and incubated o/n at 4°C with a mouse anti-TH antibody (1:1000; Santa Cruz Biotechnology), followed by 2 h RT incubation with a biotinylated goat anti-mouse secondary antibody (1:500; Millipore). Sections were then incubated with 1% avidin-biotin peroxidase complex (Vectastain ABC kit, Vector) for 30 min at RT. Staining was visualized using diaminobenzidine (DAB) solution. NAcc was identified using the mouse brain atlas (Franklin and Paxinos, 2008). Images were acquired using a brightfield microscope (Zeiss, Axiovert).

Statistical analysis

Statistical analysis of 6-OHDA lesion effects on behaviour and brain monoamine content was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA). In most cases an unpaired *t*-test was conducted. Prior to conducting a t-test, Levene's test for equality of variances was conducted; in case of non-equal variances the corresponding t-test was used and degrees of freedom adjusted accordingly. Analysis of variance (ANOVA) was conducted in cases of repeated measures, with a between-subject factor of group (6-OHDA, Vehicle) and a within-subject factor of test phase (e.g. PRS test, LH test, fear conditioning test). Where appropriate, ANOVA *post hoc* testing was conducted using the Bonferroni procedure. Statistical significance was set at $p \leq 0.05$. Where an estimate of variance is given this is the standard deviation (SD).

Results

Efficacy and specificity of 6-OHDA dopamine depletion

Four experiments were conducted investigating the behavioural effects of 6-OHDA lesion targeting the NAcc. The findings of ex vivo assessments of DA and 5-HT tissue levels in NAcc, CPu, mPFC and Amyg are presented in Table 1. Mice injected with 6-OHDA exhibited a marked (74-87%) and consistent reduction in NAcc DA in each experiment: PRS test, $t_{(14)} = 5.1$, $p<0.0005$; LNR test, $t_{(16)} = 6.8$, $p<0.0005$; LH test and treadmill test, $t_{(25)} = 10.2$, $p<0.0005$; fear conditioning test, $t_{(17)} = 10.6$, $p<0.0005$. We also assessed for effects of NAcc 6-OHDA on DA tissue levels in off-target regions, either as a specificity control or because DA function in that region could be relevant to the behavioural processes under study. Caudate-putamen was studied in each of the four NAcc experiments: there was no effect of NAcc 6-OHDA on CPu DA levels in two experiments, an increase in the PRS experiment ($t_{(14)} = -3.8$, $p<0.005$), and a decrease in the LH test + treadmill test experiment ($t_{(24)} = 3.5$, $p<0.005$). There was no effect on Amyg DA levels, as measured in two experiments. For mPFC DA, firstly it is important to note that baseline (Veh) levels were markedly lower than in NAcc, CPu and Amyg (Table 1). NAcc 6-OHDA lesions led to reduced mPFC DA levels in each of the four experiments: In the PRS and LNR experiments, DA levels were below detection in 6-OHDA mice specifically; there was a moderate-high reduction in mPFC DA in the LH test + treadmill test ($t_{(24)} = 2.8$, $p<0.01$), and in the fear conditioning test ($t_{(17)} = 4.3$, $p<0.0005$). To investigate whether this off-target effect could itself recapitulate the behavioural effects of NAcc DA depletion (see below), the effects of mPFC 6-OHDA lesion were studied directly in the LNR test and LH test. There was a marked (67-86%) and consistent reduction of mPFC DA: LNR test, $t_{(28)} = 2.5$, $p<0.02$; LH test, $t_{(22)} = 3.6$, $p<0.002$ (Table 1). Furthermore this was specific to mPFC with no impact on DA levels in NAcc, CPu or Amyg. With regard to 5-HT, in no experiment was there a reduction in 5-HT tissue levels (Table S1). To assess the effect of NAcc or mPFC 6-OHDA lesion on noradrenaline (NA) levels in NAcc, mPFC, CPu and Amyg, additional experiments were conducted and radioenzymatic assay used for NA measurements (Table S2). NAcc 6-OHDA lesion did not affect NA levels in any region; mPFC 6-OHDA lesion led to increased NA in the mPFC ($t_{(9)} = -4.6$, $p<0.002$) and decreased NA in the NAcc ($t_{(9)} = 2.4$, $p<0.05$).

Nucleus accumbens 6-OHDA efficacy and specificity was also assessed qualitatively using TH staining for DA fibre terminals. Representative 6-OHDA and Veh sections are given in Figure S3. The TH stain was markedly reduced in the NAcc core and NAcc shell of 6-OHDA mice. One additional region that exhibited reduced stain in 6-OHDA mice was the extreme medial CPu.

Table 1. Effects of NAcc or mPFC 6-OHDA infusion on tissue dopamine in brain regions of interest

			Dopamine (ng/mg tissue)							
			NAcc		CPu		mPFC		Amygdala	
Target region	Behavioural test		Veh	6-OHDA	Veh	6-OHDA	Veh	6-OHDA	Veh	6-OHDA
NAcc	Progressive ratio schedule	X	3.31	0.86^{c}	15.70	22.47^{c}	0.29	n.d.		
	(N=8 Veh, 8 6-OHDA)	SD	1.19	0.64^{c}	1.57	4.82^{c}	0.41			
NAcc	Learned non-reward	X	4.92	1.04^{c}	19.09	20.88	0.25	n.d.		
	(N=10, 9)	SD	1.64	0.52^{c}	4.73	4.44	0.24			
NAcc	Learned helplessness &	X	6.89	0.90^{c}	19.37	15.3^{b}	0.12	0.05^{b}	1.89	2.10
	Treadmill (N=13, 14)	SD	2.04	0.79^{c}	3.13	2.78^{c}	0.08	0.04^{c}	1.44	1.33
NAcc	Fear conditioned freezing	X	6.45	1.43^{c}	14.61	13.66	0.14	0.02^{c}	1.06	0.97
	(N=11, 11)	SD	1.29	0.36^{c}	2.02	2.61	0.07	0.04^{c}	0.39	0.35
mPFC	Learned non-reward	X	6.08	7.28	13.20	14.55	0.24	0.08^{a}		
	(N=13, 12)	SD	1.63	1.77	3.77	4.31	0.22	0.11^{c}		
mPFC	Learned helplessness	X	7.16	8.59	13.71	14.98	0.07	0.01^{b}	2.31	2.20
	(N=12, 12)	SD	1.48	2.66	2.79	5.21	0.06	0.01^{c}	1.54	1.19

6-OHDA dose was 2 µg per hemisphere for NAcc and 1.5 µg per hemisphere for mPFC.
Scores are mean ± SD.
a p<0.05, *b* p<0.01, *c* p<0.001 in t-tests.

Physical effects of 6-OHDA dopamine depletion

Effects of NAcc and mPFC 6-OHDA on body weight (BW) were measured for days 1 to 10 after surgery. ANOVA was conducted for post-surgery BW expressed as a percentage of pre-surgery baseline (= 100%). Data for the NAcc LH + Treadmill experiment are presented as a representative example (Figure S1): There was a Group X Day (baseline, post-surgery days 1-10) interaction ($F_{(10, 271)}$=4.3, p<0.0005); *post hoc* analysis revealed that Veh mice recovered to baseline BW by day 4 whereas 6-OHDA mice recovered by day 8, and that % BW was lower in 6-OHDA compared to Veh mice on days 1-5. For the mPFC 6-OHDA LH experiment, there was a main effect of Day ($F_{(10, 220)}$=8.8, p<0.0005) but no effect involving Group (p = 0.56); *post hoc* analysis revealed that both groups recovered baseline BW by day 4 (data not shown).

Operant tests using gustatory positive reinforcement

Progressive ratio schedule (PRS) test

The PRS test was used to investigate effects of NAcc DA depletion with 1.5 or 2.0 µg 6-OHDA on motivation for sucrose pellets in non-food deprived mice under effortful conditions. Following operant training and prior to surgery, mice were given three PRS tests, and for group allocation were counter-balanced on total number of pellets earned (Veh: 47.9 ± 0.3, 1.5 µg: 45.8 ± 0.3, 2 µg: 46.2 ± 0.8 (p=0.48)) and final ratio attained (Veh: 28.4 ± 0.4, 1.5 µg: 27.4 ± 0.4, 2 µg: 27.4 ± 1.0 (p=0.62)). For analysis of 6-OHDA effects on cumulative number of pellets earned and PR ratio attained, the PRS test was divided into four 10-min blocks and ANOVA was conducted for Dose X Time-block. For cumulative pellets earned there was a main effect of Dose ($F_{(2, 21)}$=4.20, p<0.03; Fig. 1A); *post hoc* analysis revealed that mice injected with 2 µg 6-OHDA earned less pellets than Veh mice (p<0.04).

There was also a main effect of Dose for PR ratio attained ($F_{(2, 21)}$=3.9, p<0.04; Fig. 1B); 2 µg 6-OHDA mice attained a lower ratio than Veh mice (p<0.04). The reduced motivation of NAcc DA-depleted mice was also evident in mean post-reinforcement pause (PRP) ($F_{(2, 21)}$=15.4, p<0.0005; Fig. 1C) with both 1.5 µg and 2 µg 6-OHDA mice exhibiting higher mean PRP than Veh mice, and in peak response rate (PRR) ($F_{(2, 21)}$=3.5, p<0.05; Fig. 1D) which was lower (and occurred at a lower ratio) in 2 µg 6-OHDA mice than in Veh mice.

Behavioural effects of DA depletion in all other tests were studied using 2 µg 6-OHDA, specifically.

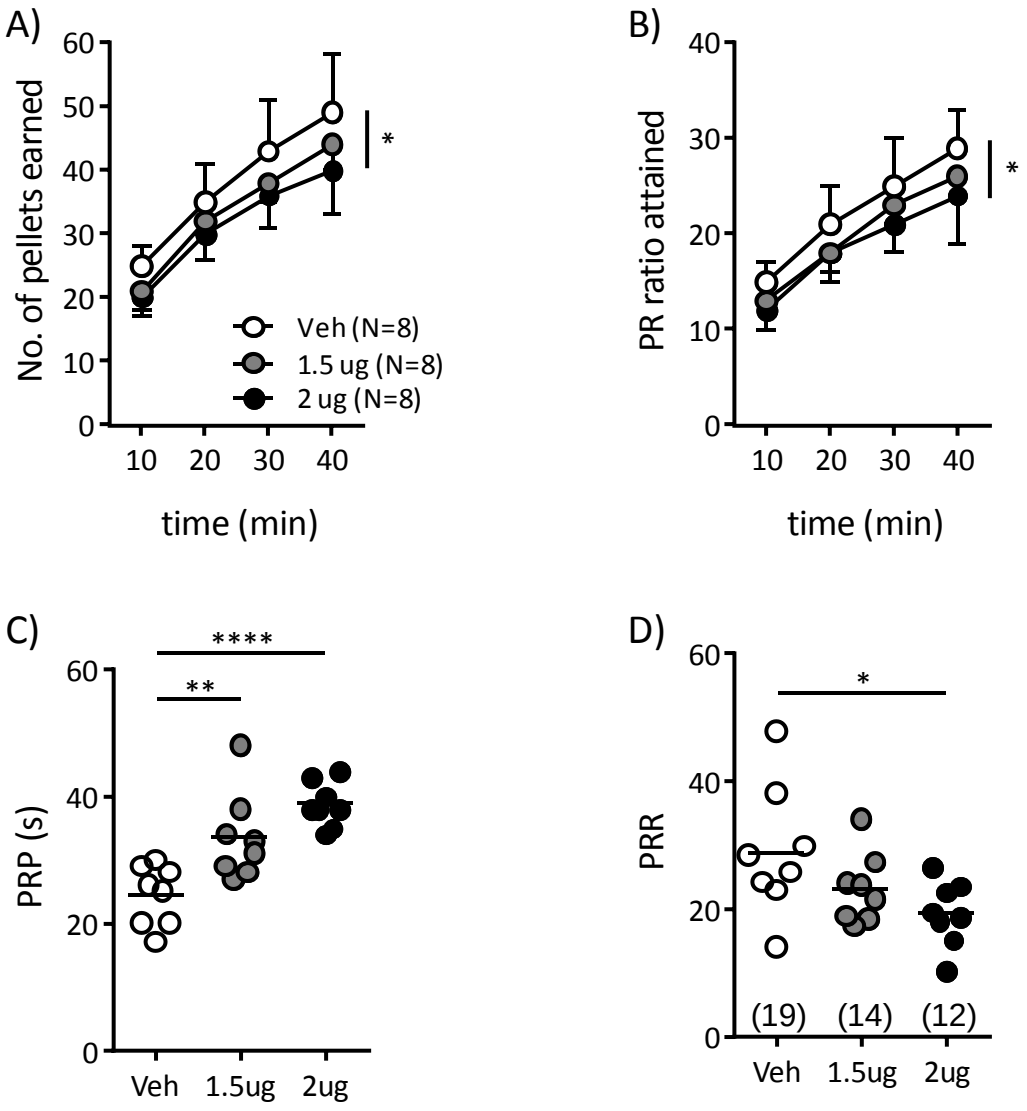

Figure 1. Effects of NAcc DA decrease (Veh, 1.5 µg, 2 µg 6-OHDA) on behavioural measures in the progressive ratio schedule (PRS) test. A) Mean ± SD cumulative scores for number of pellets earned at 10, 20, 30 and 40 min. B) Mean+/-SD cumulative scores for ratio attained at 10, 20, 30 and 40 min. For each measure there was a main effect of dose in 2-way ANOVA, and *indicates p<0.05 for 2ug 6-OHDA vs Veh in *post hoc* Bonferroni test. C) Scatterplot and mean for post-reinforcement pause (PRP, seconds (s)). D) Absolute peak response rate (PRR). Values in parentheses indicate the ratio at which the PRR was achieved. For each measure there was a dose effect in 1-way ANOVA, and * p<0.05, ** p<0.001, *** p<0.005 in *post hoc* Bonferroni tests.

Learned non-reward (LNR) test

The LNR test was used to investigate effects of NAcc or mPFC DA depletion on cognitive reward-directed behaviour. Mice were tested on three consecutive stages of both simple discrimination (SD) and learned non-reward (LNR) (SD+LNR 1-3) prior to DA depletion, and therefore any effects of the latter were unlikely to be due to impaired task acquisition. That mice acquired the task was indicated by improved performance across successive stages. For example, for total errors there was a main effect of Stage for both simple discrimination ($F_{(2, 54)}$ = 3.7, p<0.04) and learned non-reward ($F_{(2, 38)}$ = 4.0, p<0.03) (Fig. 2A); the decrease in total errors across stages indicated that mice learned rule-switching (Nilsson et al., 2012; Tait and Brown, 2007). The total error scores at LNR3 were used to counterbalance allocation of mice to NAcc 6-OHDA and Veh groups. At the post-DA depletion simple discrimination stage (SD4), there was no effect of NAcc DA depletion on any measure, e.g. total errors (p=0.37, Fig. 2B). In contrast, NAcc DA depletion did impair learned non-reward behaviour at LNR4 (Fig. 2B), in the form of increased omissions to initiate a trial ($t_{(9.5)}$ = -2.3, p<0.05), increased incorrect choice responses ($t_{(17)}$ = -3.2, p<0.005) and increased total errors ($t_{(12.3)}$ = -2.8, p<0.02). There was no effect on omissions to make a choice response (p<0.2) or correct choice responses (p<0.6).

In the mPFC experiment, as for the NAcc, performance improved across successive stages, e.g. total errors decreased across stages of simple discrimination ($F_{(2, 29)}$ = 17.8, p<0.0005) and learned non-reward ($F_{(2, 29)}$ = 6.9, p<0.01). At the post-DA depletion simple discrimination stage (SD4) there was no effect on any measure, e.g. total errors: Veh 16.7 ± 25.5, 6-OHDA 17.8 ± 18.1 (p=0.90). In contrast to the NAcc experiment, there was also no effect of mPFC DA depletion on learned non-reward behaviour at LNR4, e.g. total errors: Veh 69.6 ± 43.6, 6-OHDA 89.7 ± 59.7 (p=0.34).

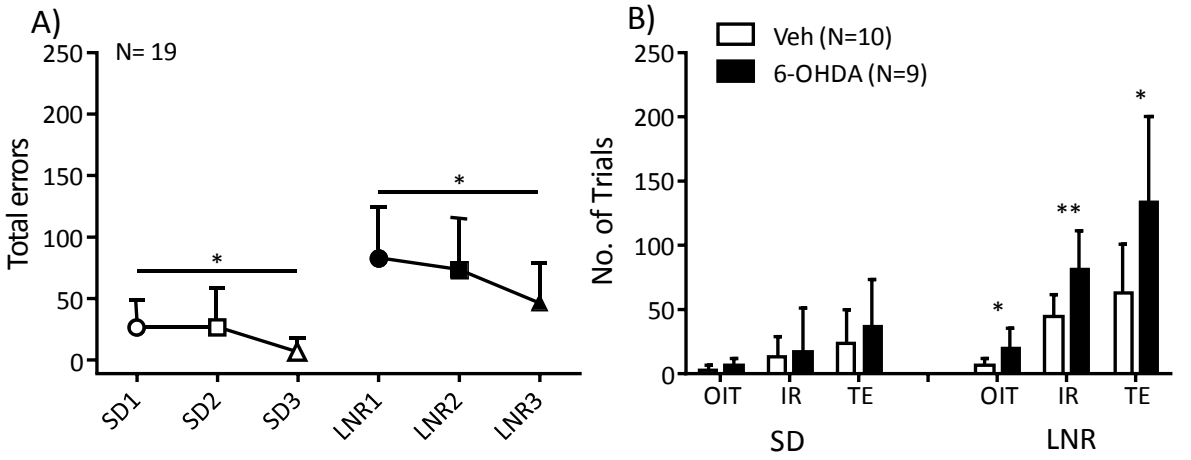

Figure 2. Effects of NAcc DA decrease (Veh, 2 µg 6-OHDA) on behavioural measures in the spatial discrimination (SD) and learned non-reward (LNR) stages of the LNR test. A) Pre-lesion training: mean+SD scores for total errors in stages SD1-3 and stages LNR1-3 prior to NAcc 6-OHDA lesion; mice were allocated to Veh and 6-OHDA groups by counter-balancing on LNR3 total-error scores. For both SD and LNR there was a stage effect in 1-way ANOVA and * indicates p<0.05 for stage 3 vs Stage 1 in post hoc Bonferroni tests. B) Post-NAcc 6-OHDA lesion testing: mean+SD scores in SD4 and LNR4 tests for omissions to initiate a trial (OIT), incorrect choice responses (IR) and total errors (TE). In the LNR test specifically there was an effect of NAcc DA depletion in t-tests, * p<0.05, ** p<0.01.

Operant tests using footshock negative reinforcement

Learned helplessness (LH) test

The LH test was used to investigate effects of NAcc or mPFC DA depletion on learning, motivation and emotion in mice exposed to inescapable and subsequently escapable footshock stimuli. A habituation session, three inescapable footshock pre-exposure sessions, and an escape test, were conducted on consecutive days. In the NAcc experiment, in the habituation session, DA depletion led to a decrease in locomotor activity ($t_{(25)}$ = 3.7, p<0.001, Fig. 3A) and an increase in % time freezing (Veh: 3.2 $\pm$ 1.5 %, 6-OHDA 6.3 $\pm$ 2.5 %, $t_{(25)}$ = -3.8, p<0.001). Mice were then exposed to three daily sessions of inescapable footshock. During the final session, there was no effect of DA depletion on locomotor activity (Veh: 4617 $\pm$ 1148 a.u./min, 6-OHDA 3717 $\pm$ 1291 a.u./min, p=0.08), but DA depleted mice did exhibit increased mean % time freezing relative to Veh mice (Veh: 10.8 $\pm$ 5.3 %, 6-OHDA 18.7 $\pm$ 12.0 %, $t_{(25)}$ = 3.4, p<0.03). In the Escape test conducted on the following day, there was no effect of DA depletion on locomotor activity (p=0.46, Fig. 3B), or % time freezing (Veh: 11.8 $\pm$ 5.3 %, 6-OHDA 13.6 $\pm$ 7.9 %, p=0.49). Dopamine depletion resulted in increased mean latency to escape ($t_{(17.8)}$ = -2.1, p<0.05, Fig. 3C) and increased failure to escape ($t_{(25)}$ = -2.2, p<0.05, Fig. 3D). Escape failures were also analysed in blocks of 10 trials to investigate for DA-depletion learning effects: there was a main effect of group ($F_{(1, 25)}$ = 4.6, p<0.05) and also of trial block ($F_{(1, 50)}$ = 4.4, p<0.02); whilst both 6-OHDA and Veh mice exhibited increased escape responses in trials 21-30 versus 1-10, indicating learning, the relative deficit in DA-depleted mice persisted (Fig. 3E). The escape deficit in DA-depleted mice co-occured with decreased footshock reactivity ($t_{(23)}$ = 2.5, p<0.05, Fig. 3F, one outlier per group was removed for analysis). We further investigated the finding that locomotor activity was reduced by NAcc DA depletion in the habituation session (Fig. 3A). This might have indicated a motor impairment (albeit temporary) or, given that the arena was novel to the mice during this session, increased anxiety/decreased motivation to explore the novel environment. An additional experiment was conducted in which mice underwent a habituation session prior to 6-OHDA NAcc lesion, and were then re-tested in the familiar environment. Under these conditions there was no effect of NAcc DA depletion on locomotor activity (p=0.17, Fig. S2) thereby supporting the interpretation of increased anxiety/decreased motivation to explore the novel arena.

In the mPFC experiment, there was no effect of DA depletion on locomotor activity in the habituation session (Veh: 8583 $\pm$ 1442 a.u./min, 6-OHDA 8856 $\pm$ 1924 a.u./min, p=0.70) or on % time freezing (Veh: 2.0 $\pm$ 1.6 %, 6-OHDA 2.7 $\pm$ 2.1 %, p=0.36). During the final session of inescapable footshock, there was again no effect on locomotor activity (Veh: 4636 $\pm$ 945 a.u./min, 6-OHDA: 4249 $\pm$ 1111 a.u./min, p=0.37) or % time freezing (Veh: 6.2 $\pm$ 3.5 %, 6-OHDA: 9.2 $\pm$ 5.7 %, p=0.13). Also in the Escape test, mPFC DA depletion was without behavioural effects: locomotor activity (Veh: 5653 $\pm$ 1170 a.u./min, 6-OHDA: 5736 $\pm$ 1436 a.u./min, p=0.88); mean % time freezing (Veh: 5.9 $\pm$ 3.5 %, 6-OHDA: 6.8 $\pm$ 3.7 %, p=0.57); escape failures (Veh: 9.5 $\pm$ 7.8, 6-OHDA: 13.1 $\pm$ 9.9, p=0.34); mean escape latency (Veh: 3.2 $\pm$ 1.0 s, 6-OHDA: 3.5 $\pm$ 1.1 s, p=0.48); footshock reactivity (Veh: 163 $\pm$ 49 a.u./s, 6-OHDA: 149 $\pm$ 57 a.u./s, p=0.53)..

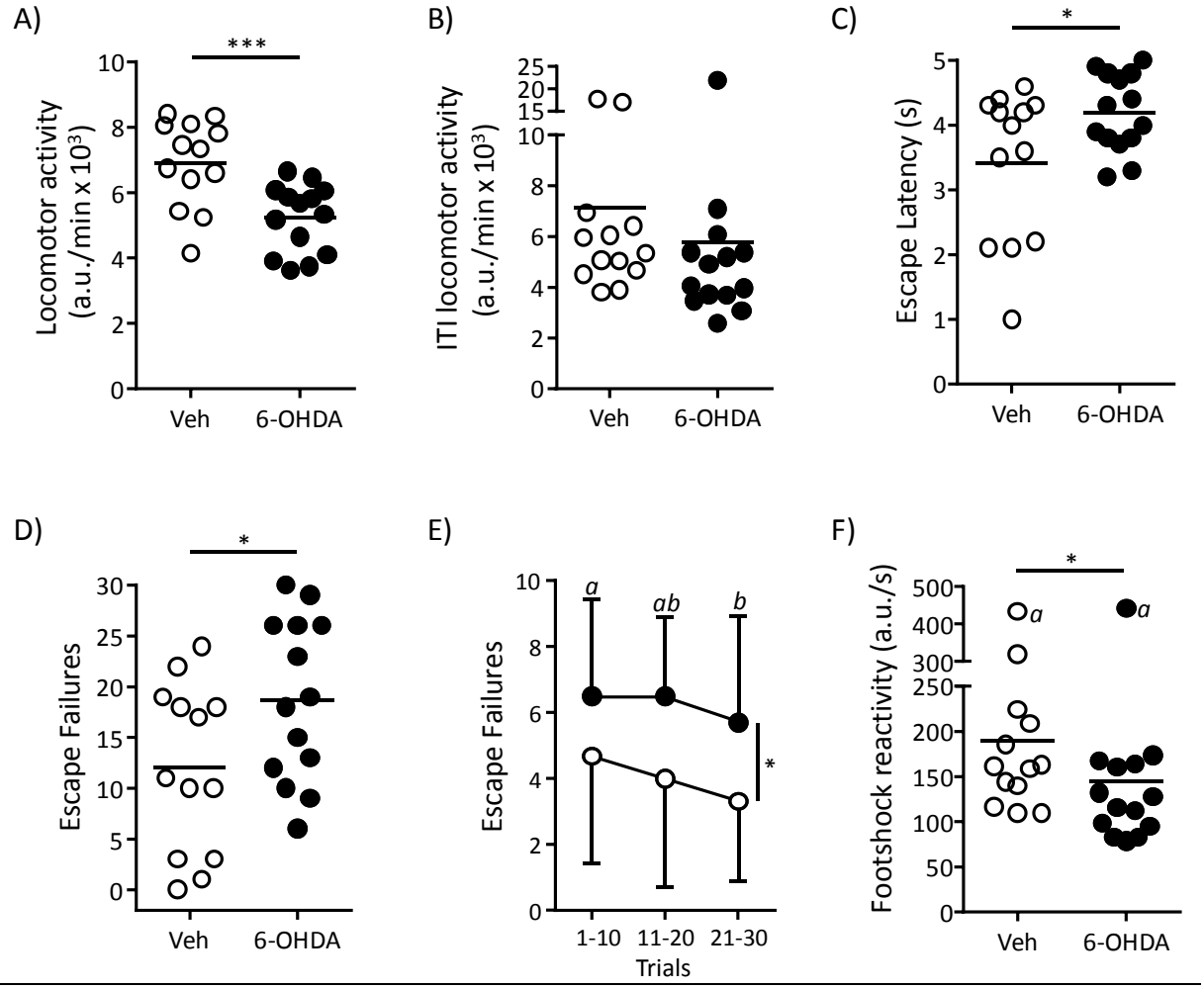

Figure 3. Effects of NAcc DA decrease (Veh (N=13), 2 µg 6-OHDA (N=14)) on behavioural measures in the learned helplessness (LH) paradigm. A) Locomotor activity in the habituation session on day 1. B) Locomotor activity during intervals between footshocks in the escape test on day 5. C) Mean escape latency to 30 footshocks in day 5 escape test (maximum footshock = 5 s). D) Total escape failures in day 5 escape test. E) Escape failures per 10-trial block of escape test. F) Footshock reactivity in terms of mean distance/s in day 5 escape test. In A-D and F, * $p<0.05$, *** $p<0.001$ in t-tests. In E, there were significant main effects of group and block in 2-way ANOVA; * $p<0.05$ for group, and letters indicate pairwise block effects in post hoc Bonferroni tests. In F), a indicates outlier values (according to Grubbs' test) that were excluded for the t-test.

Treadmill test

The treadmill test was used to investigate effects of NAcc DA depletion on effortful avoidance of and escape from an aversive stimulus. Mice are first exposed to the apparatus when it is stationary and then at walking speed so that they can acquire operant avoidance and escape responses. The NAcc DA depleted and Veh mice that were studied in the treadmill test had been tested 2 days previously in the LH paradigm. In the treadmill training session, there was no effect of DA depletion on total footshock time ($p=0.44$, Fig. 4A). The next day, during the 5-min pre-test session at a treadmill speed of 20 cm/s, there was a borderline non-significant increase in total footshock time in DA depleted mice (Veh: 5.8 ± 8.4 s, 6-OHDA: 12.0 ± 8.3 s, $p=0.06$). In the treadmill test, conducted at a speed of 23 cm/s, the running time achieved was decreased in DA depleted compared to Veh mice ($t_{(25)} = 3.7$, $p<0.001$, Fig. 4B). Ten out of 13 Veh mice achieved the maximum running time of 20 min without accumulating 10 s of footshock, whereas this was the case in only 1

of 14 6-OHDA mice. Accordingly, total footshock time was higher in DA depleted mice ($t_{(21.4)}$ = -4.1, p<0.001, Fig. 4C).

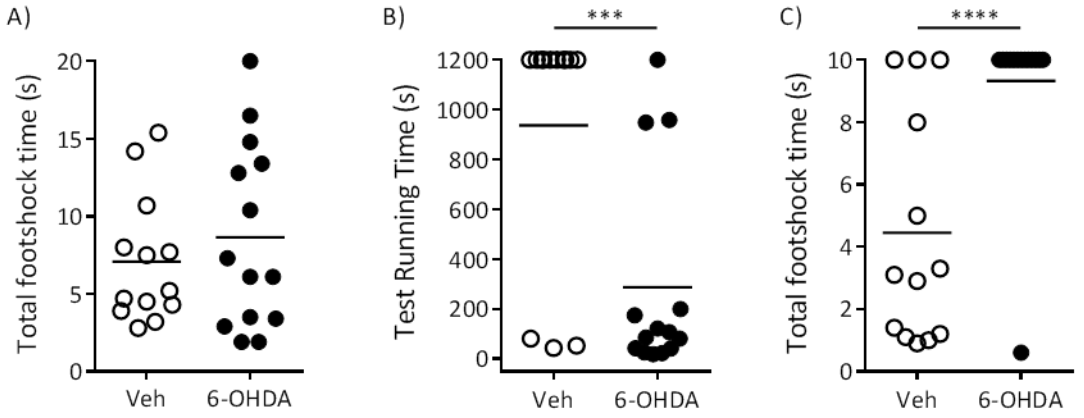

Figure 4. Effects of NAcc DA decrease (Veh (N=13), 2 µg 6-OHDA (N=14)) on measures at the training and test session of the treadmill test. Mice were the same as those studied in the learned helplessness test. A) Total duration of footshock received during the treadmill training session. B) Running time achieved during the test session with maximum duration of 20-min (1200 sec). C) Total duration of footshock received during the test session, with maximum cumulative duration of 10 sec. *** p<0.001, **** p<0.0001 in t-tests.

Pavlovian fear conditioning test

Auditory tone-footshock conditioning, measured as % time spent freezing, was used to investigate effects of NAcc DA depletion on aversive stimulus-stimulus learning. During the CS-US conditioning session, there was a main effect of CS trial-block on % time freezing ($F_{(2, 34)}$ = 23.9, p<0.0005, Fig. 5A), with acquired freezing increasing continuously across trials. There was no main or trial interaction effect of DA depletion (p=0.54, Fig. 5A). On the following day, consecutive expression tests were conducted for contextual and CS fear-freezing. There was no effect of NAcc DA depletion on % time freezing in either the context test (p=0.32; Fig. 5B) or the CS test (p=0.51, Fig. 5C).

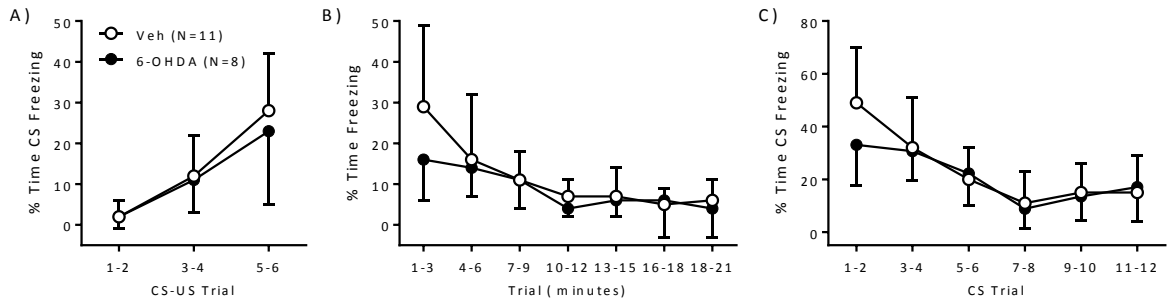

Figure 5. Effects of NAcc DA decrease (Veh. 2 µg 6-OHDA) on % time spent freezing (mean±SD) in the CS-US fear conditioning paradigm. A) Freezing acquisition during CS-US conditioning, B) Freezing expression to context, and C) Freezing expression to the CS. There were no significant effects of NAcc DA depletion.

Hot plate test

Nucleus accumbens DA depletion resulted in reduced active behavioural responsiveness to footshock in the LH test and the treadmill test. One possible explanation for these effects is analgesia. To investigate this, in mice that had been studied in the NAcc LH + Treadmill experiment or

the NAcc fear conditioning experiment, the hot plate test was carried out to assess thermal pain sensitivity. There was no effect of DA depletion in either mice studied previously in the LH test + Treadmill test (response latency: Veh: 28.4 $\pm$ 11.8 s, 6-OHDA: 26.6 $\pm$ 10.5 s, p=0.68), or in mice studied in the fear conditioning test (Veh: 33.1 $\pm$ 11.0 s, 6-OHDA: 27.5 $\pm$ 3.8 s, p=0.12). Also in mPFC DA depleted mice that had been studied in the LH test, there was no effect of DA depletion (Veh: 24.8 $\pm$ 7.3 s, 6-OHDA: 22.8 $\pm$ 9.6 s, p=0.25).

Discussion

The aims of the present study were to investigate the effects of 6-OHDA-induced DA depletion in the nucleus accumbens on reward and punishment processing in a mouse test battery, to thereby establish: (1) whether the effects of this manipulation as demonstrated comprehensively in rat also apply in mouse; and (2) to what extent accumbens-dopamine modulation of motivational processes, in particular, underlie deficiencies in both positive- and negative-stimulus processing. Bilateral DA and DA fibre depletion in the nucleus accumbens resulted in reduced responding for gustatory reward on a progressively effortful operant schedule, and reduced responding to a rewarded operant stimulus that was previously non-rewarded. Furthermore, it decreased two-way escape responding to an aversive stimulus experienced as uncontrollable, and decreased one-way active avoidance-escape responding to an aversive stimulus that was controllable by effort. Whilst NAcc 6-OHDA injection also resulted in a moderate level of chronic mPFC DA depletion, direct mPFC DA depletion did not recapitulate the behavioural effects of NAcc DA depletion, as demonstrated for the LNR test and LH test. There were also no/only minor effects of 6-OHDA on DA levels in caudate putamen and amygdala. Owing to co-administration of drugs blocking their respective transporter proteins, neither serotonin nor noradrenaline were depleted by NAcc 6-OHDA lesion, indicating that the observed effects were consequent to DA depletion. mPFC 6-OHDA lesion did lead to moderately increased NA in mPFC and decreased NA and increased 5-HT in the NAcc, and these effects call for some caution in accepting the evidence for no effect of mPFC DA depletion.

<u>Accumbens DA deficiency impairs reward motivation under effortful or ambiguous conditions</u>

The progressive ratio schedule with gustatory reward has been applied previously to study effects of NAcc DA depletion in rat: reduced motivation was demonstrated specifically for high-effort PR schedules (Assadi et al., 2009; Hamill et al., 1999). To our knowledge the present study provides the first demonstration that, also in mice, NAcc DA depletion reduces motivation for gustatory reward under the high-effort conditions of a PR schedule. These effects were to some extent 6-OHDA dose-dependent, and the higher, more efficacious dose of 2 µg/hemisphere was the specific dose used in all other experiments. Mice were maintained at 100% baseline body weight (BW) for the PRS test, with the aim of maximising the palatable incentive value of sucrose pellets relative to their calorific value; rat studies have also conducted PRS testing at or close to baseline BW (e.g. (Hamill et al., 1999)). Given that mice received extensive operant training and three pre-depletion PRS sessions, there is little likelihood that the deficits in DA-depleted mice were due to impairment in task acquisition. Our findings support the hypothesis that NAcc DA modulates specific motivational processes underlying behaviour directed towards palatable reward (and probably also other reward categories), including behavioural activation, exertion of effort, appetitive behaviour, and sustained task engagement (Salamone and Correa, 2012). Sucrose (vs. water)-preference studies in rats demonstrate that NAcc DA depletion does not decrease reward wanting under non-effortful conditions (Berridge and Robinson, 1998). In human depression, patients do not differ from healthy controls in their reports of the intensity and pleasantness of sucrose (Dichter et al., 2010). However,

using monetary reward, depressed patients exhibit reduced motivation to expend effort to obtain reward (Treadway et al., 2012).

The learned non-reward test deployed here was first described for mice relatively recently (Nilsson et al., 2012); the absolute and relative (LNR versus SD stages) number of trials and errors to attain learning criteria, and rate of learning-set across stages, were similar in the present study to those in the original report (Nilsson et al., 2012). Two-choice spatial discrimination (SD) requires acquisition of CS-reward and CS-non-reward associations, and the elicitation of appetitive and avoidance behaviours, respectively, by these two associations. The LNR contingency change requires relearning that the CS-non-reward association is now a CS-reward association and concomitant overcoming of avoidance due to learned non-reward (LNR). Accumbens DA depletion was without effect on both omission and commission errors during SD, indicating that the CS-US acquisitions and appropriate appetitive and avoidance behaviours were intact. At the LNR stage, NAcc DA depleted mice exhibited increases in omission of trial initiation and commission of incorrect responses, each of which we interpret as indicating an increased state of LNR. That is, in the absence of normal NAcc DA levels, motivation - e.g. behavioural activation, effort, approach - relative to a CS-US reward with a recent history of non-reward was decreased. Mice received extensive operant training and three pre-depletion SD+LNR sessions, so the high LNR errors in DA-depleted mice were not due to impairment of task acquisition. In a rat study with a reversal learning (rather than LNR) test, DA depletion in dorsal striatum impaired reversal (Fanous et al., 2007). Also in a rat reversal learning study, NAcc injection of D_1R or D_2R antagonist or D_1R agonist were each without effect, and a D_2R agonist impaired reversal learning (Haluk and Floresco, 2009). Excitotoxic lesion of the NAcc, particularly the core region, has been demonstrated to impair reversal between strategies and stimulus dimensions, as well as reversal between stimuli within a dimension; however, the use of excitotoxic lesion means that the observed effects cannot be unequivocally attributed to DA deficiency (Reading and Dunnett, 1991). In the original mouse LNR study, systemic serotonin 2C receptor ($5\text{-}HT_{2C}R$) antagonism decreased LNR errors specifically (Nilsson et al., 2012). Given that $5\text{-}HT_{2C}R$ antagonism increases mesocorticolimbic DA function (Di Matteo et al., 2001), this performance-enhancement effect is relevant to the current findings that NAcc DA depletion leads to performance deficiency.

A major projection region of the NAcc, and one which is also important in reversal learning and other forms of behavioural flexibility, is the PFC (Clarke, 2004). In rat, the orbital PFC is important in simple reversal learning and the medial PFC in reversal learning between stimulus dimensions (Brown and Bowman, 2002). In the present study, mPFC DA depletion was without effect on either SD or LNR. Blockade of D_1R or D_2R in mPFC in rat reduced behavioural flexibility; it acted primarily by increasing perseveration (Floresco et al., 2006), which is not tested in the LNR paradigm. It is thus possible that in reversal learning tasks, mPFC contributes mainly to overcoming perseveration rather than to LNR (Allen and Leri, 2014; Hsiao et al., 2013). Furthermore, neurotransmitters other than DA, in particular serotonin, may modulate mPFC-dependent cognitive flexibility (Clarke, 2004; van der Plasse and Feenstra, 2008).

<u>Accumbens DA deficiency impairs aversion-control motivation under previously uncontrollable or effortful conditions</u>

We established the learned helplessness effect paradigm for mice in order to have an objective test for aversive un/controllability in this species (Pryce et al., 2012). Following repeated sessions of matched escapable footshock (ES) or inescapable footshock (IS), when both groups are tested under ES conditions, the IS mice exhibit more escape failures. This deficit in escape behaviour is underlain by decreased motor reactivity to the footshock, which we have interpreted as a

motivational deficit, particularly given that pain sensitivity remains intact (Pryce et al., 2012). In the present study we restricted the experiment to the IS condition, and demonstrated that NAcc DA depletion resulted in increased escape failure concomitant with increased escape latency and decreased motor reactivity to the aversive US. In rat, 6-OHDA injection in the striatum led to increased operant escape failure in subjects exposed to IS, and the same effect was obtained when 6-OHDA was injected into the substantia nigra pars compacta (SNC) (Winter et al., 2007). Also in rat, in the absence of prior IS, 6-OHDA NAcc DA depletion decreased operant (lever press) escape-avoid behaviour; furthermore, in intact subjects, performing this operant behaviour led to increased NAcc DA release (McCullough et al., 1993). Rats exposed to IS and exhibiting a high level of 2-way escape failure were characterised by a decrease in the proportion of active DA neurons in the VTA (Belujon and Grace, 2015). Therefore, in addition to chronic NAcc DA depletion impairing motivational processes that mediate engagement with reward, as evidenced in PRS and LNR tests, the current mouse LH findings add to the evidence that depleted NAcc DA also impairs murine motivational and learning processes mediating disengagement from punishment (Wenzel et al., 2015). Indeed, deficient motivation has been proposed to be one of the three major mediators of the LH effect (Maier and Seligman, 1976; Pryce et al., 2011b), and the current evidence indicates that decreased NAcc DA underlies this state. The other proposed major mediators of the LH effect are increased emotional responding to aversive stimuli perceived as uncontrollable, and decreased cognitive expectancy that stimuli will be controllable (Maier and Seligman, 1976; Pryce et al., 2011a). The proportion of time spent freezing provides a measure of emotionality in the LH paradigm: freezing was increased in NAcc DA depleted mice relative to control mice during IS pre-exposure but not during the escape test; freezing is also not increased in IS relative to ES intact mice (Pryce et al., 2012). By separating the escape test session into blocks of trials, we were able to analyse for learning effects on escape performance; both Veh and NAcc DA depleted mice exhibited reduced escape failures across the session, indicating that impaired operant learning did not underlie the deficit in the latter group. In rat, the mPFC has been demonstrated to be critical to the learning of un/controllability and is probably a region in which the cognitive expectancy of stimulus control is processed: thus, GABA-mediated (picrotoxin) disinhibition of mPFC resulted in increased escape behaviour in subjects exposed to IS (Amat et al., 2008). In the present study, there were no effects of mPFC DA depletion on behaviour of mice in the LH test, suggesting that DA does not modulate mPFC-mediated cognitive expectancy aspects of the LH effect.

The footshock treadmill test allows for the assessment of active avoidance-escape from an aversive stimulus under effortful conditions. NAcc DA depletion resulted in a marked decrease in running time. The majority of DA-depleted mice ran for only 1-2 minutes of the test session until receiving the maximum footshock amount. These data suggest that NAcc DA depletion decreased the absolute level of effort mice are able to/are motivated to exert to actively avoid-escape footshock. The findings complement the evidence from the LH test that NAcc DA depletion decreased motivation to escape footshock following IS. Whilst it is important to note that the treadmill experiment was conducted with the same mice that were studied in the LH test, the majority of vehicle mice showed typical treadmill behaviour, i.e. they completed the 20-min test, indicating the absence of a major confounding effect from the LH test. Previous studies have used the footshock treadmill test to assess various factors, including physical exercise (Massett, 2005) and inflammation (Carmichael et al., 2006). It has been proposed as a readout test for fatigability, a core symptom of depression (ICD-10, DSM-5). As noted above, the current findings suggest that NAcc DA depletion acts more to reduce absolute level of effort rather than cumulative effort to avoid-escape aversion. Given the complexity of the psychopathological construct of fatigue, both of these states could be

components of it in depression. Thus, fatigue is proposed to comprise muscular, motivational, motor, emotional and cognitive components (Demyttenaere et al., 2005; Kluger et al., 2013). It has been proposed that deficient DA-ergic modulation of striatal and cortical regions is an important pathophysiological factor for fatigue (Demyttenaere et al., 2005; Salamone et al., 2007).

The third test in which effects of NAcc DA depletion on responsiveness to aversive stimuli were studied was Pavlovian fear conditioning. In contrast to the operant tests, there was no effect on each of CS-US acquisition, context expression and CS expression, as measured using freezing behaviour. In rat, evidence for whether exposure to inescapable CS-US elicits increased extracellular DA in the NAcc is equivocal, with different findings being accommodated by proposing NAcc sub-region specificity (Levita et al., 2002b; Wilkinson et al., 1998). Excitotoxic lesioning of the rat NAcc core was without effect on CS-US acquisition or expression or on context-US acquisition, but did reduce context expression of conditioned fear (Levita et al., 2002a). Therefore, the previous rat and current mouse findings indicate that the NAcc is not a major contributor to the mesolimbic DA regulation of Pavlovian fear conditioning, with the amygdala being a more likely region of DA action (Pezze and Feldon, 2004; Wenzel et al., 2015). Thus, of the three tests that exposed mice to footshock, in the two tests where active operant behaviour resulted in negative reinforcement, NAcc DA depletion led to a deficit in this behaviour, and in the test where behaviour was without consequence, NAcc DA depletion was without behavioural effect.

Conclusion and Outlook

The present study provides novel evidence for mice that reduced DA levels in NAcc lead to reduced responding to: stimuli that predict reward under effortful conditions, stimuli that now predict reward but with recent association with non-reward, an aversive stimulus recently experienced as uncontrollable, an aversive stimulus under effortful conditions. The findings obtained with the current operant test battery extend rat findings for NAcc DA function to mouse, and also add to the overall understanding of the importance of NAcc DA as an important modulator of motivational processes - including behavioural activation, exertion of effort, sustained task engagement - elicited by both rewarding and aversive stimuli (Bromberg-Martin et al., 2010; Salamone and Correa, 2012; Wenzel et al., 2015). This study also adds to the evidence that motivational and motor functions of DA, classically attributed to the VTA-mesolimbic and SNC-nigrostriatal pathways, respectively, are intimately inter-related and can both be affected by NAcc DA deficiency (Haber, 2014; Sesack and Grace, 2010). Our rationale for establishing the present mouse test battery was to enable the integrated study of motivational processing of rewarding and aversive stimuli. It can then be applied to investigate whether specific manipulations induce deficits in one or more of these processes that are relevant to motivational domains in psychopathology. The evidence that NAcc DA deficiency impairs reward motivation under effortful or ambiguous conditions suggests its relevance to the RDoC of Positive valence systems, the core symptom of loss of interest-pleasure in depression and the core negative symptoms in schizophrenia. The evidence that the same manipulation impairs aversion-control motivation under previously uncontrollable or effortful conditions suggests its relevance to the state of helplessness and the core symptom of fatigue, in depression. That the battery enables detection of effects of a single manipulation on motivational processing of both reward and aversion indicates that it will facilitate the study and understanding of how reward and aversion processing are inter-related. We readily acknowledge that 6-OHDA DA depletion is a severe manipulation with little aetiological validity. Nonetheless, mouse environmental manipulations with aetiological validity, such as the chronic social defeat stressor, do indeed impact on DA signalling (Azzinnari et al., 2014; Chaudhury et al., 2013; Lammel et al., 2014; Tanaka et al.,

2012), and also lead to impaired aversion-control motivation in the two-way avoid-escape and the treadmill test (Azzinnari et al., 2014) and impaired reward motivation in the PRS test and LNR test (Bergamini et al., in prep-b). Future studies can combine aetiologically valid genetic and environmental manipulations with the reward-aversion test battery presented here, to identify the pathophysiological processes underlying motivational deficits, discover novel treatment targets, and screen the preclinical therapeutic efficacy of compounds developed for these targets.

Supplementary information

Learned non-reward (LNR) test: Stages of training and testing

Operant behaviour training for the LNR test was based on that described by Nilsson et al.(Nilsson et al., 2012)

Nosepoke training. Only the central stimulus nose-poke port was used. Each trial started with switching on the houselight and the LED in the stimulus nose-poke port. A nosepoke led to the stimulus port LED switching off and delivery of a sucrose pellet into the feeder port at the opposite wall. Collection of the pellet initiated a 5 s timeout. Maximum number of rewards was 40, and criterion was ≥ 30 rewards in each of two consecutive sessions.

Trial training. The mouse had to nosepoke in the feeder port to start each trial, activating the houselight and the central stimulus port LED. A nosepoke in the stimulus port led to the stimulus port LED switching off and delivery of a sucrose pellet into the feeder port. A limited response time was introduced at two stages of each trial: there was a limited hold of 10 s for a feeder port nosepoke to initiate each trial; once the trial was initiated, there was a limited hold of 6 s for a nosepoke in the stimulus port. Omission of either response led to termination of the trial and onset of the next 5 s timeout. To meet criterion, mice had to complete ≥ 49 correct nosepokes over a total of 70 trials. After attaining criterion on one session, two further sessions were given, one for the left and one for the right stimulus ports, with order counterbalanced across subjects.

Spatial discrimination (SD). The left and right stimulus ports were available in the testing apparatus and the central stimulus port was replaced by a blank panel. The correct stimulus port was the opposite to that which was used for the last session of *trial training*. A response in the correct stimulus port (correct choice responses, CR) led to switching off the LED in both stimulus ports, pellet delivery in the feeder port and onset of the 5 s timeout after pellet collection. A response in the incorrect stimulus port (incorrect choice response, IR) led to onset of the 5 s timeout. Failure to either self-initiate a trial within 10 s (omissions to initiate a trial, OIT) or to nosepoke in either of the two stimulus ports within 6 s (omissions to make a choice response, OR), led to termination of the trial and immediate onset of the timeout. Therefore, for each trial, the mouse response was either correct (CR), incorrect (IR) or an omission (OIT, OR). Each SD session comprised seven blocks of 10 trials each. The session criterion for SD learning was nine correct responses in any 10-trial block.

Learned non-reward (LNR). The incorrect stimulus port at SD was now correct, the correct stimulus port at SD was replaced by a black panel and a third stimulus port, not available during SD, became the incorrect stimulus port. Otherwise, conditions were as for SD.

Table S1. Effects of NAcc or mPFC 6-OHDA infusion on tissue serotonin in brain regions of interest

			Serotonin (ng/mg tissue)							
			NAcc		CPu		mPFC		Amygdala	
Target region	Behavioural test		Veh	6-OHDA	Veh	6-OHDA	Veh	6-OHDA	Veh	6-OHDA
NAcc	Progressive ratio schedule	X	0.58	0.76	0.17	0.18	0.37	0.33		
	(N=8 Veh, 8 6-OHDA)	SD	0.25	0.31	0.08	0.08	0.32	0.23		
NAcc	Learned non-reward	X	0.56	0.57	0.29	0.35	0.09	0.05		
	(N=10, 9)	SD	0.22	0.13	0.09	0.17	0.05	0.02		
NAcc	Learned helplessness &	X	1.00	0.95	0.87	1.30	0.30	0.54 *a*	1.39	1.60
	Treadmill (N=13, 14)	SD	0.39	0.27	0.45	1.92	0.11	0.38	0.24	0.60
NAcc	Fear conditioned freezing	X	0.85	0.94	0.23	0.27	0.47	0.42	1.23	1.14
	(N=11, 11)	SD	0.24	0.25	0.06	0.07	0.10	0.09	0.36	0.23
mPFC	Learned non-reward	X	0.77	0.90	0.27	0.34	0.39	0.40		
	(N=13, 12)	SD	0.22	0.33	0.10	0.10	0.09	0.14		
mPFC	Learned helplessness	X	0.77	1.14 *b*	0.27	0.28	0.18	0.17	1.29	1.15
	(N=12, 12)	SD	0.20	0.34	0.07	0.11	0.14	0.07	0.34	0.19

6-OHDA dose was 2 µg per hemisphere for NAcc and 1.5 µg per hemisphere for mPFC.
Scores are mean ± SD.
a p<0.05, *b* p<0.01.

Table S2. Effects of targeted 6-OHDA infusion on tissue noradrenaline in brain regions of interest

		Noradrenaline (pg/mg tissue)							
		NAcc		CPu		mPFC		Amygdala	
Target region		Veh	6-OHDA	Veh	6-OHDA	Veh	6-OHDA	Veh	6-OHDA
NAcc	X	13.22	15.65	2.60	3.58	15.81	11.53	20.19	18.23
(N=5 Veh, 9 6-OHDA)	SD	10.29	9.75	0.68	1.68	4.45	5.51	8.02	5.68
mPFC	X	6.96	4.65 *a*	4.72	5.34	8.73	19.63 *b*	15.77	15.88
(N=5, 8)	SD	2.22	0.87	1.11	1.52	2.48	4.77	8.20	6.41

6-OHDA dose was 2 µg per hemisphere for NAcc and 1.5 µg per hemisphere for mPFC.
Scores are mean ± SD.
a p<0.05, *b* p<0.001.

Figure S1

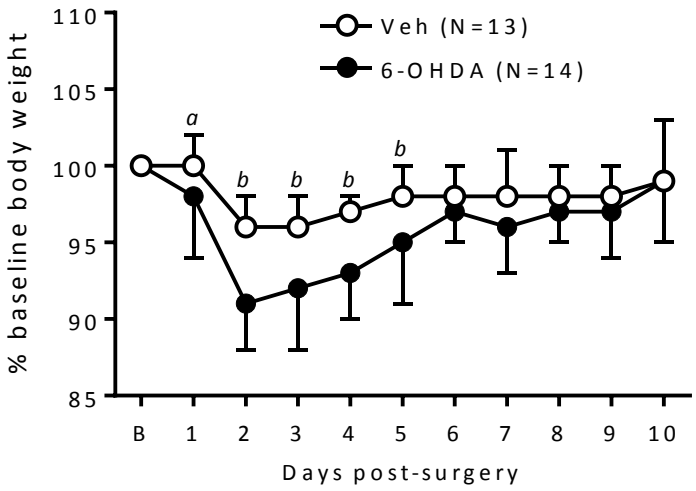

Figure S1. Effect of NAcc DA decrease (Veh, 2 µg 6-OHDA) on post-surgery body weight expressed as a percentage of pre-surgery baseline (= 100%). Scores are % mean +/- SD scores. In 2-way ANOVA there was a group x day interaction, and *a* p<0.05, *b* p<0.001 for day-specific 6-OHDA vs Veh effects in *post hoc* Bonferroni tests.

Figure S2

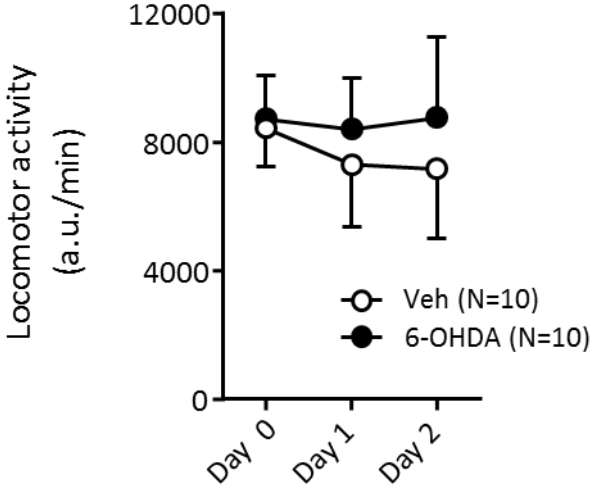

Figure S2. Lack of effect of NAcc DA decrease (Veh, 2 µg 6-OHDA) on post-surgery locomotion in a familiar arena. Day 0: locomotor activity was measured in a novel arena (same as that used for the learned helplessness test). Twelve days after 6-OHDA NAcc lesion or NAcc Veh, mice were re-tested for locomotor activity in the same arena, on two consecutive days (Day 1 and 2). Scores are mean +/- SD.

Figure S3

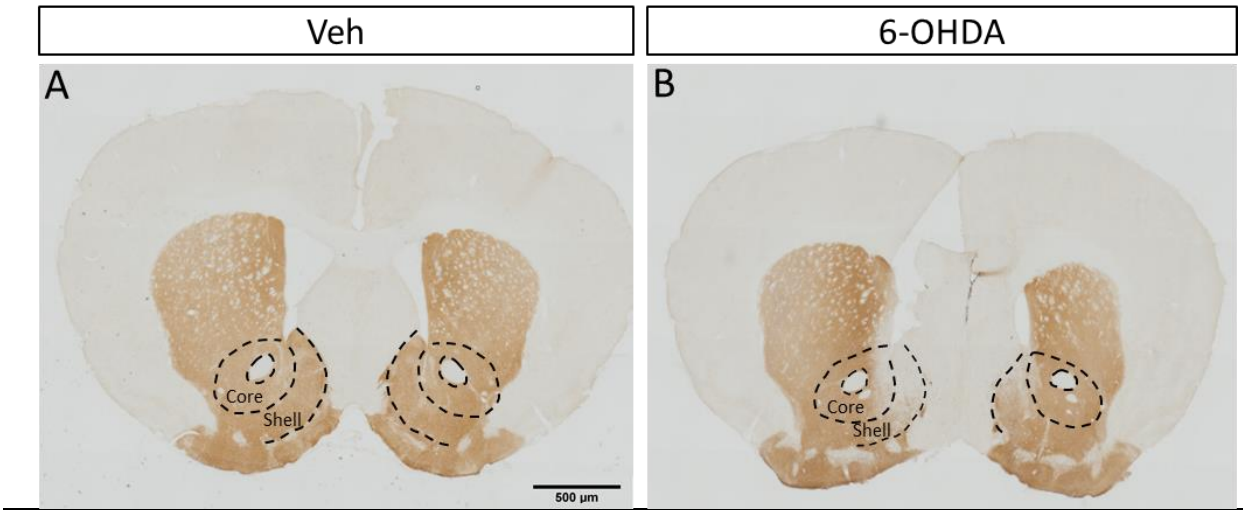

Figure S3. Immunohistochemical staining for tyrosine hydroxylase to delineate the extent of the depletion of dopaminergic fibres following (A) Vehicle or (B) 6-OHDA injection in the nucleus accumbens. Brains were perfused at day 12 post-injection. Coronal images of mouse striata showing loss of TH^+ fibers in the NAcc core and shell following injection of 6-OHDA.

Study B

Mouse chronic social stress induces peripheral and CNS inflammation, dopamine deregulation and disrupted reward processing

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* Giorgio Bergamini designed the study, carried out the experiments and data analysis, and wrote the manuscript

Abstract

Stressful life events are risk factors for depression. Stress induces, and depression is associated with, immune activation. Core depression symptoms, most notably decreased motivation, are proposed to stem from dopamine (DA) dysfunction. Whilst it is proposed that DA dysfunction can result from immune activation, the evidence for this is currently sparse. We have previously shown that exposure of mice to a chronic uncontrollable psychosocial stressor, chronic social defeat (CSD), increases fear conditioning, helplessness and fatigue, and induces peripheral immune activation. The aims of the present study were to investigate CSD effects on: (1) Immune function in periphery and brain at cellular and molecular levels, including the mesolimbic DA pathway. (2) Mesolimbic DA pathway function. (3) Reward-directed behaviour using operant tasks. Relative to controls, CSD mice exhibited increased spleen levels of granulocytes ($CD11b^{+}/SSC^{hi}/Ly6C^{int}$), inflammatory monocytes ($CD11b^{+}/SSC^{lo}/Ly6C^{hi}$) and T helper 17 cells ($CD4^{+}/IL\text{-}17A^{+}$), increased plasma levels of iNOS, and increased liver expression levels of genes for kynurenine-pathway enzymes. In the ventral tegmental area, microglia activation (Iba1, *Cd11b*) was increased, and in nucleus accumbens (NAcc), dopamine turnover (DOPAC/DA) was decreased. When challenged with a DA reuptake inhibitor, CSD mice exhibited both attenuated hyper-locomotion and NAcc *c-Fos* activation. In operant tests with sucrose-pellet reinforcement, CSD mice exhibited decreased motivation under effortful conditions. This study provides some of the most robust evidence to-date in support of the hypothesis that stress-induced peripheral and brain inflammation co-occur with attenuated mesolimbic DA function including decreased interest in reward.

Introduction

Major depressive disorder (MDD, hereon referred to as depression) is common and complex with respect to symptoms and aetio-pathophysiology (Nabeshima and Kim, 2013). Depression-related psychopathology includes marked deficits in reward-related behavioural domains (Russo and Nestler, 2013). Indeed, in depressed patients, there is evidence for reduced appetitive motivation (Sherdell et al., 2012) and reward learning (Pizzagalli et al., 2005). Moreover, reward-related deficits have been associated with reduced activation of striatal regions (Pizzagalli, 2014; Pizzagalli et al., 2009). It has been hypothesized that dysfunction of the mesolimbic DA system is a major pathophysiology underlying reduced interest in reward in depression (Dunlop and Nemeroff, 2007; Pizzagalli, 2014).

Stressful life events are key proximal risk factors for depression (Kendler et al., 1999; Slavich et al., 2009). Psychosocial stressors that are uncontrollable, including interpersonal loss (e.g. of status), defeat and submission are particularly depressogenic (Gilbert, 1992; Kendler et al., 2003). Pre-clinical studies in rodents have investigated various stressors (Krishnan and Nestler, 2011), including social stress (Azzinnari et al., 2014; Krishnan et al., 2007). In chronic social defeat (CSD), mice are exposed continuously to aggressive dominant mice including short daily attack. Their submissive behaviour is ineffective in controlling attacks by a different aggressor mouse each day (Azzinnari et al., 2014; Kudryavtseva et al., 1991), such that CSD mice experience chronic exposure to uncontrollable social stressors. Mouse CSD induces a number of depression-relevant behaviours, including decreased sucrose preference and social interaction (Covington et al., 2009), and increased fear acquisition, helplessness and fatigue (Azzinnari et al., 2014).

Clinical and preclinical studies have demonstrated stressor reactivity of the immune system. (Slavich and Irwin, 2014). In humans, psychosocial stress activates the sympathetic nervous system (SNS), and release of epinephrine and norepinephrine and binding by adrenergic receptors on immune cells promotes an inflammatory cascade (Bierhaus et al., 2003). Positive correlation between chronic social stressors and levels of C-reactive protein, interleukin (IL)-6 (Gruenewald et al., 2009) and soluble receptor for tumor necrosis factor-α, have been reported (Chiang et al., 2012). In mice, repeated social defeat induces brain trafficking of myeloid cells and microglial activation (Wohleb et al., 2011; Wohleb et al., 2013). In CSD mice, splenomegaly and increased plasma levels of the pro-inflammatory cytokines TNF-α and IL-6 have been reported (Azzinnari et al., 2014).

There is growing interest in the hypothesis that stress-induced immune-inflammation contributes to depression pathophysiology (Beumer et al., 2012). Activation of the immune system in depression is certainly well-documented (Maes, 2011). Depressed patients show higher blood levels of the inflammatory markers CRP, IL-6, IL-1 and TNF-α (Muller, 2014). In brain, the neuroinflammatory marker translocator protein (TSPO) was increased in the anterior cingulate cortex (ACC) and correlated with depression severity (Setiawan et al., 2015). Normalization of inflammatory markers after successful antidepressant treatment has been described (Hannestad et al., 2011a), as has treatment resistance in patients with increased inflammatory markers at treatment onset (O'Brien et al., 2007). One consequence of increased pro-inflammatory cytokine levels is the upregulation of the tryptophan enzyme indoleamine 2,3 dioxygenase (IDO) in macrophages, and synthesis of kynurenine pathway metabolites (Campbell et al., 2014; Schwarcz et al., 2012). These include 3-hydroxykynurenine (3-HK), a free radical generator that promotes oxidative stress (Schwarcz et al., 2012), and the glutamate receptor agonist and neurotoxin quinolinic acid (QA) (Dantzer and Walker, 2014). There is evidence for activation of the kynurenine pathway in depression (Savitz et al., 2015b; Steiner et al., 2011). Furthermore, it has been proposed that kynurenine pathway over-activity can impact on DAergic function (Felger and Miller, 2012; Felger et al., 2013; Miller et al., 2013). Indeed, experimental stimulation of the immune system in healthy

humans induces reduced reward interest, fatigue, and psychomotor retardation, consistent with impaired DA function (Felger and Miller, 2012). Rodent studies have demonstrated the importance of the dopamine neurons located in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc) in the modulation of reward learning and motivation (Russo and Nestler, 2013). Using neurotoxin to specifically deplete DA projections to the NAcc, we have demonstrated that decreased NAcc DA levels attenuate motivation for gustatory reward in a simple but effortful operant test and in a learned non-reward test requiring cognitive flexibility (Bergamini et al., Submitted manuscript).

Against the above background, the overall aim of the present study was to apply the mouse CSD stressor to investigate for co-occurrence of: (1) activated immune-inflammatory responses in the periphery and brain, with a focus on VTA and NAcc in the latter. (2) Reduced DA function in the VTA-NAcc pathway, including pharmacological challenge. (3) Reduced operant responding for reinforcement in tests that are sensitive to NAcc DA status. Demonstration that a psychosocial stressor impacts on the VTA-NAcc DA pathway at immune, neurobiological and behavioural levels would constitute some of the most robust evidence to date for the psycho-neuro-immune hypothesis of depression.

Materials and methods

Animals

Breeding of C57BL/6J mice was conducted in-house. Male offspring were weaned at age 3 weeks and caged in groups of 2-3 littermates. Mice were aged 10-13 weeks and weighed 24.0-30.0 g at study onset. Male CD-1 mice (Janvier, Saint-Berthevin, France) were aged 8 months, were ex-breeders, and caged singly at study onset. Mice were maintained on a reversed 12:12 h light-dark cycle (lights off 07:00-19:00 h) in an individually-ventilated caging system at 21-22°C and 50-60% humidity. Complete-pellet diet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were available ad libitum, unless stated otherwise below for operant training and testing. In the week prior to CSD, all C57BL/6J mice were handled and weighed on five days. All procedures were conducted under a permit for animal experimentation (No. 170/2012) issued by the Veterinary Office, Zurich, Switzerland, in accordance with the Animal Protection Act (1978) Switzerland.

Chronic social defeat

Three days prior to onset of 15-day chronic social defeat (CSD), mice were tested in terms of locomotion in a 15-min activity test, and activity scores were used to counter-balance the allocation of mice to CSD and control (CON) groups. This, and the CSD procedure, was conducted as described previously (Azzinnari et al., 2014). Briefly, social defeat sessions were conducted under dim light on 15 consecutive days. Cages contained a divider made from transparent Plexiglas and perforated with multiple holes for sensory interactions. On day 1, each CSD C57BL/6J mouse was removed from its home cage and placed in the home cage of a CD-1 mouse. Mice remained together until either a cumulative total of 60-s physical attack had occurred or 10 min had elapsed. The CSD mouse remained in the compartment in which it had been attacked and the CD-1 mouse was placed in the opposite compartment, allowing continuous olfactory, visual and auditory contact during the following 24 h. On day 2, the CSD mice x CD-1 mice pairings were rotated so that each CSD mouse was placed in the home cage of and confronted with a novel CD-1 mouse, and vice versa. To prevent bite wounds - an essential aspect of the method given that a major aim of the study was to investigate psychosocial stress-induced inflammation - the lower incisors of CD-1 mice were trimmed

at 3-day intervals using a rodent tooth-cutting forceps under brief isoflurane anaesthesia across CSD. Control (CON) mice remained in littermate pairs and were handled and weighed daily.

Experimental design

Eight separate experiments were conducted with the overall and integrative aim of studying the effects of CSD on peripheral and central inflammation, DA structure-function, and reward-directed behaviour (Figure 1). In the first experiment, CSD effects on spleen and whole brain immune-cell status at day 16 were assessed using flow cytometry. In the second experiment, day 16 liver levels of genes encoding enzymes important in the kynurenine pathway were measured using RT-PCR, and plasma levels of iNOS were measured by ELISA. Third, brains were perfused for immunohistochemistry of the microglia marker Iba1 in VTA and additional brain regions, at day 16. Fourth, gene expression of the microglia marker Cd11b and of genes central to DA synthesis and transport were measured in VTA tissue punches using RT-PCR. Fifth, the tissue level of DA and its major metabolite were measured in NAcc using HPLC-ED. In the sixth experiment, CSD effects on responsiveness to a DA reuptake inhibitor in terms of an activity-test (day 16) and home-cage activity (day 16-17) and NAcc immediate-early gene expression (day 17), were investigated. In the seventh experiment, mice were first given baseline testing in a two-bottle saccharin versus water test and then the effects of CSD were tested at day 16. In the final experiment, mice were first trained in an operant apparatus with sucrose-pellet reinforcement, exposed to CSD or CON, and then CSD effects were assessed on reward motivation at day 8 and on behaviour in a learned non-reward task at days 16-29.

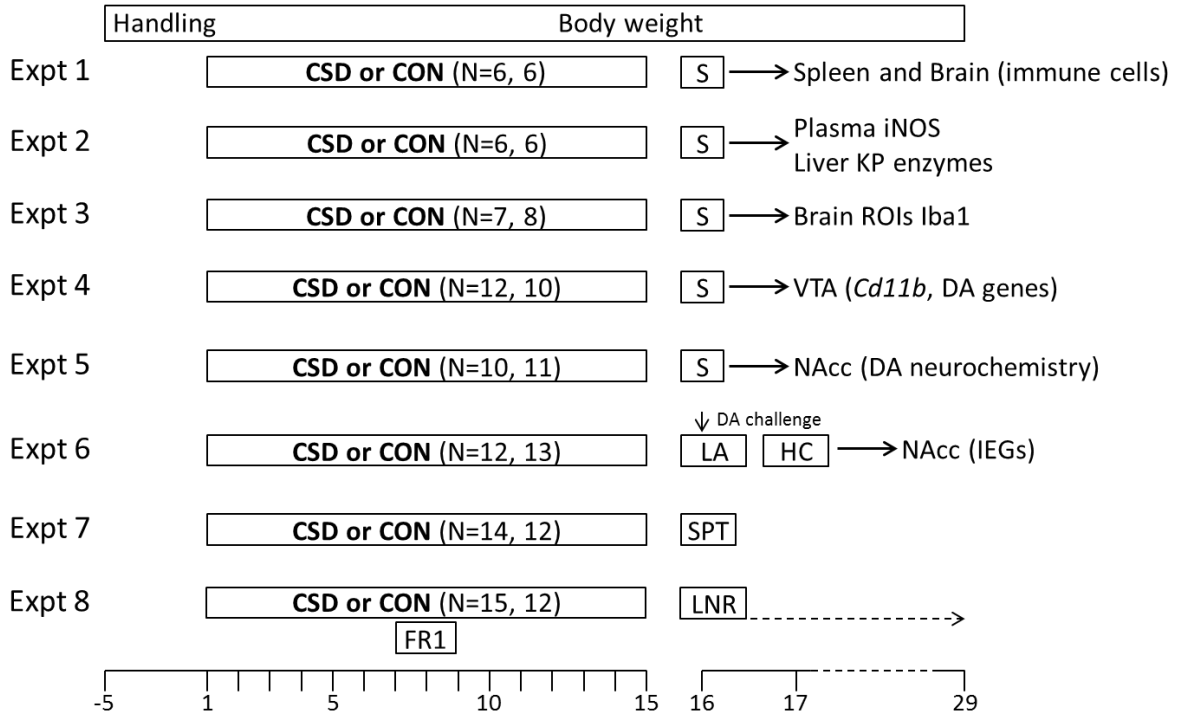

Figure 1. Experimental designs. Eight experiments were conducted with mice exposed to chronic social defeat (CSD) or control handling (CON) on days 1-15. Mice were handled prior to experiments and body weights were measured daily. Experiment 1: brain and spleen samples (S) were collected on day 16. Immune cells were analysed using flow cytometry. Experiment 2: plasma liver samples were collected on day 16 for assessment of gene expression of kynurenine pathway (KP) enzymes. Experiment 3: brains were perfused on day 16 and processed for immunohistochemical analysis of Iba1 expression in several brain regions of interest (ROIs). Experiment 5: on day 16 brains were collected and NAcc punches were dissected for HPLC analysis of DA and metabolites. Experiments 6: on day 16 mice were injected with the DA reuptake inhibitor GBR 12909 or VEH,

and their locomotor activity (LA) was monitored for 2 hours. Afterwards, mice activity was monitored in the home cage (HC). On day 17 mice were injected with GBR 12909 or VEH and their brains were collected for qPCR analysis of immediate-early genes (IEGs). Experiment 7: saccharin preference test (SPT) was performed on day 16. Experiments 8: an operant test on a fixed ratio 1 (FR1) schedule for sucrose reward was conducted on day 8 and a learned non-reward (LNR) test was conducted on days 16 to 29.

Isolation of splenocytes and brain mononuclear cells and flow cytometry

For isolation of splenocytes, the spleen was minced in Roswell Park Memorial Institute (RPMI) medium (5% fetal calf serum) and digested for 20 min at RT in Hanks' balanced salt solution (HBSS) containing 50 µg/ml DNase I and 100 µg/ml Collagenase/Dispase (Roche). The digestion was passed through a 100 µl nylon mesh (BD Biosciences), followed by lysis of erythrocytes in Ammonium-Chloride-Potassium (ACK) buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). After a final resuspension in HBSS, cells were counted and 5 x 10^6 were used for antibody staining. Mononuclear cells (MNCs) from entire brain were isolated as described previously (Moransard et al., 2010; Suter et al., 2003). Briefly, mice were perfused transcardially with HBSS, the brain dissected out, chopped with a scalpel, and digested for 30 min at 37°C in HBSS containing 50 µg/ml DNase I and 100 µg/ml Collagenase/Dispase. The digestion was passed through a 100 µl nylon mesh and re-suspended in 30% Percoll (Sigma). The gradient was centrifuged at 29,000 g for 30 min at 4°C. The interphase containing mononuclear cells was collected and washed with HBSS. For both spleen and brain cells, each sample was divided into two halves. One was used for extracellular staining and the other for extracellular+intracellular staining. Intracellular staining of cytokines was performed after a 4 h incubation with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and Ionomycin (500 ng/ml) (Sigma), to allow accumulation of cytokines within cells.

For flow cytometry of splenocytes and brain MNCs, cells were re-suspended in FACS buffer comprising 2% FCS, 5 mM EDTA and 0.01% NaN_3 in PBS. Fc receptors were blocked by incubation with anti-mouse CD16/32 (Fc-block, BD Pharmingen). The LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular Probes) was used to exclude non-viable cells. For extracellular staining, the antibodies used were: PE-Cy5.5 CD45 (BD Pharmingen), PE-Cy7 CD11b (BD Pharmingen), FITC Ly6c (BioLegend) and PE MHC II (BD Pharmingen). For extra- and intracellular staining, the antibodies used were: PE-Cy5.5 CD45 (BD Pharmingen), PE-Cy7 CD11b (BD Pharmingen), APC-Cy7 CD4 (BD Pharmingen), PE-Cy5 Foxp3 (eBioscience), PE IL-17A (BD Pharmingen), Alexa 700 CD8 (eBioscience), APC Ki67 (*BD* Pharmingen), FITC TNF (BD Pharmingen) and eF450 IFNγ (eBioscience). To assess the activation status of immune cells the proinflammatory cytokines TNF and IFNγ and the major histocompatibility complex (MHC) class II were used. Cell proliferation was assessed using the marker Ki-67. For brain MNCs, absolute cell counts were achieved using fluorescent Counting Beads (Molecular probes). Expression levels of proteins were estimated using the geometric mean of fluorescence intensity (MFI). Flow cytometry was conducted with a LSR II Fortessa (BD Biosciences). FlowJo software (Tree Star Inc.) was used for flow cytometry data analysis.

Plasma iNOS ELISA

Mice were decapitated and trunk blood was collected in EDTA-coated tubes (Microvette 500 K3E, Sarstedt) and placed on ice. Tubes were centrifuged at 3000 rpm for 15 min at 4°C and plasma aliquots were transferred to cryotubes (Protein LoBind tubes, Eppendorf) and stored at -80°C. Plasma iNOS was measured in duplicate using a commercial ELISA kit according to the manufacturer's instructions (Mouse Inducible nitric oxide synthase ELISA Kit, MyBioSource).

Real time PCR assays

Mice were decapitated and brain, spleen and liver, depending on experiment, were dissected out and frozen on dry-ice. Frozen brains were sectioned coronally at 1.0 mm intervals using a stainless-steel brain matrix, and regions of interest, identified with reference to a mouse brain atlas (Franklin and Paxinos, 2008), were micro-dissected bilaterally using a brain punch (∅ = 1.0 mm) (Azzinnari et al., 2014). Brain punches were placed in 350 µl of lysis RLT buffer (Qiagen) (containing 1% β-mercaptoethanol) and homogenized using a tissue lyser (Mixer-Mill 300, Qiagen) with stainless steel beads (∅ = 5 mm, Retsch). Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen). For spleen and liver, total RNA was isolated using Trizol (Ambion) according to the manufacturer`s instructions, and further digested with DNase I (Fermentas). The list of genes of interest and the respective primers used are given in Table S1. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative gene expression analysis was performed using SYBR green (Applied Biosystems) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). PRC cycle conditions were: 2 min at 50°C, 2 min at 95°C; 40 cycles: 15 s at 95°C, 30 s at 60°C, 30 s at 72°C. A melting curve was run for each PCR plate. Threshold cycle (Ct) values of the target gene were normalized to the Ct values of the corresponding reference gene, using the ΔΔCt method described by (Pfaffl et al., 2002). For brain and spleen, A*ctb* was used as the reference gene, and for liver the geometric mean of Ct values for *Hprt1* and *Tbp* was used as normalization factor (NF). Relative expression values were $\log_2$-transformed for statistical analysis.

HPLC-ED of NAcc tissue dopamine

Mice were decapitated and the brain dissected out, frozen on powdered dry-ice and stored at -80°C. Frozen brains were sectioned coronally at 1.0 mm intervals and the NAcc was micro-dissected bilaterally using a brain punch (∅ = 1 mm) (see previous section for details). The two punches were weighed and homogenized together, using 250 µl ice-cooled perchloric acid (0.4 M). Ultra-sonication was conducted for 5 s at 30% power (VibraCell, VCX130PB, Sonics and Materials, Inc., Newtown CT, USA), followed by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was passed through a 0.22 µm filter (Minisart RC4, Sartorius AG, Göttingen, Germany) and kept on ice until analysis. High performamce liquid chromatography (HPLC) and electrochemical detection (ED) were conducted for DA and its major metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), according to (Oeckl et al., 2012). Briefly, isocratic separation of DA was carried out with a reversed-phase C18 column (YMC-Pack ODS-AQ, 100×2.1 mm, S-3 µm, YMC Europe GmbH, Dinslaken, Germany). The mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium salt, 1.0 mM $Na_2EDTA \times 2H_2O$, 8.0 mM NaCl, 100 mM $NaH_2PO_4 \times 2H_2O$ (pH 3.80), mixed with 9.3% acetonitrile, and was delivered at a flow rate of 0.4 ml/min. For ED an electrochemical cell with a glassy carbon electrode and an ISAAC Ag/AgCl reference electrode (VT-03, Antec, Zoeterwoude, Netherlands) was used. Homogenates (20 µl) were injected onto the HPLC system using an autosampler (ASI-100T). Dopamine, DOPAC and HVA concentrations were calculated using an external standard calibration and expressed as ng/mg brain tissue.

Iba1 immunohistochemistry

Mice were deeply anesthetized with pentobarbital and perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) and fixative (4% paraformaldehyde (PFA) in PBS). Brains were dissected out, post-fixed over-night in 4% PFA and cryoprotected in 30% sucrose for 48h, frozen on powdered dry ice and stored at -80°C until sectioning. Brains were sectioned coronally at 40 µm using a microtome (Zeiss), with sections collected in antifreeze solution (15% glucose and 30% ethylene

glycol in 50 mM phosphate buffer, pH 7.4) and stored at -20°C. Free-floating sections were washed with PBS, quenched with 3% H_2O_2 for 15 min, blocked with 2% normal serum and incubated o/n at 4°C with a rabbit anti-Iba1 antibody (1:1000; Wako Chemicals) followed by a 2 h RT incubation with a biotinylated goat anti-rabbit secondary antibody (1:500; Millipore). Sections were then incubated with 1% avidin-biotin peroxidase complex (Vectastain ABC kit, Vector) for 30 min at RT. Labeling was visualized by using diaminobenzidine (DAB) solution. The brain regions of interest (ROIs) were VTA, NAcc, amygdala and medial prefrontal cortex. Sections including these regions, 3-6 per mouse, were identified with the aid of a mouse brain atlas (Franklin and Paxinos, 2008). Images were acquired using a brightfield microscope (Axiovert, Zeiss). Digital image analysis (DIA) of Iba1 staining was performed blind with respect to mouse identity using the software ImageJ (NIH): the threshold for positive staining was determined and used to calculate the percentage of the area of each image that was Iba1-immunoreactive (IR) (Wohleb et al., 2011).

Behavioural tests

Baseline activity testing and effects of GBR 12909

In each experiment, at 3 days prior to onset of CSD/CON, a 15-min activity test in a novel arena was conducted and total locomotor distance was used to allocate mice to CSD and CON groups in a counter-balanced manner. An automated test apparatus was used (Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany (Pryce et al., 2012)).

In the DA pharmacological challenge experiment, on day 16, CSD and CON mice were injected intra-peritoneally (i.p.) with either the dopamine transporter (DAT) inhibitor GBR 12909 dihydrochloride (Sigma-Aldrich) at 6mg/10ml/kg in physiological saline vehicle or with vehicle only. After return to the home cage for 30 min, mice were placed into the same arena as used for baseline activity testing but now containing a central divider with an opening (“gate”) via which mice could transfer from one side of the arena to the other, to stimulate activity/exploration, for 2 h. The main measure of interest was total locomotor activity (arbitrary units) per 2 h. Mice were then returned to their home cage and activity was monitored continuously for 24 h using a passive infrared sensor located above the cage (MP Motion Sensor AMN12112, Panasonic) and connected to a RedLab recoding unit (1024HLS, Meilhaus Electronic). For this purpose, CON pairs were separated from each other using the same type of divider used to separate CSD from CD1 mice; they were habituated to the divider for 2 h per day on two days prior to this home cage activity measurement. On day 17, after completion of the home-cage activity monitoring, mice were injected with a second i.p. dose of GBR 12909 or vehicle and after 30 min were decapitated and the brains processed for NAcc RNA extraction (see Real time PCR assays).

Two-bottle saccharin test

For this experiment, mice were habituated to drinking from 15 ml screw-top polypropylene tubes from which the tip had been removed. Two such tubes were attached adjacently to the cage lid each day at 08:00 h and removed at 16:00 h. Amount drunk per tube in ml was calculated based on weight. On days 12 to 8 prior to onset of CSD/CON, the pairs of mice were presented with one tube containing water and one tube containing 0.15% (W/V) saccharin (as sodium salt hydrate, Sigma) solution, with the left-right positioning of the two tubes alternated across days (Cathomas et al., 2015). On the final day of baseline measurements, average saccharin consumption per mouse was 5.4 $\pm$ 1.4 g, average water consumption was 0.15 $\pm$ 0.10 g, and average saccharin preference was

98.7 ± 0.5% (mean±SD). The effects of CSD on saccharin and water drinking and saccharin preference were tested in a single test at day 16.

Operant reward tests

Operant apparatus

Operant training and testing were conducted using operant boxes (TSE Systems GmbH, Bad Homburg, Germany) details of which are given elsewhere (Ineichen et al., 2012). Briefly, nose-poke ports detected mouse nose poke responses via an infra-red beam, and port position and number changed according to the test (see below). Sucrose pellets (14 mg, Dustless Precision Pellets, TSE Systems GmbH) were delivered singly into the feeder port, signalled by a tone from a speaker. Pellet retrieval was detected via infra-red beam.

Feeding protocols

Mice had ad libitum access to food until age 11 weeks. At age 11 weeks, for 5 days, daily body weight (BW) and daily food intake were measured to obtain mean free-feeding (baseline) values. Beginning the following week and continuing throughout operant training, mice were food restricted and kept at 90-95% of baseline BW, to ensure adequate motivation during operant training with sucrose pellet reinforcement. For one week prior to and during the 15-day CSD, mice were given sufficient normal diet to return to and maintain 100% baseline BW. In accordance with our previous observations, CSD mice required a greater amount of daily food to maintain this target BW than did CON mice (see Results). Mice were maintained at 95% baseline body weight throughout LNR testing, with CSD mice again requiring an increased amount of normal diet relative to CON mice.

Fixed-ratio 1 schedule of reinforcement (FR1) test and learned non-reward (LNR) test

The same operant training was required for the FR1 and LNR tests, and details are given in *Supplementary information* of Study A. Briefly, mice were trained to initiate each trial by one operant nosepoke into the feeder port, which resulted in illumination of a stimulus nose-poke port in the opposite, stimulus wall. One response in the stimulus port activated signalled delivery of a sucrose pellet into the feeder port, which the mouse returned to and retrieved the pellet. At 2.5 s after pellet retrieval, the next trial could be initiated. In the final stage of training, mice were allowed 10 s to initiate each trial with a single nosepoke in the feeder port and, if they did so, 6 s to make a single nosepoke in the stimulus port; omission of either response resulted in the onset of a 5 s timeout. The criterion for completion of training was 2 sessions of 70 trials at each of which ≥ 49 pellets were earned under the above conditions. When mice had attained training criterion on these two sessions they were tested in two further daily sessions. The mean number of pellets earned during these four sessions was used to counter-balance the allocation of mice to CSD and CON groups.

The FR1 test comprised the same conditions as used at the final stage of training (described above), and was conducted at CSD day 8 with mice maintained at 100% baseline BW. Measures of interest were: omissions to initiate trials at the feeder port, omissions to emit responses at the stimulus port in initiated trials, total pellets earned, and session duration.

The LNR test was based on that described by Nilsson et al. (2012). For this test there were three spatial locations (left, middle, right) for a nose-poke port in the stimulus wall. The first stage of the test was a two-choice spatial discrimination (SD): following trial initiation, the mouse had to choose between two stimulus nose-poke ports whilst a blank panel occupied the third location. A response in the correct stimulus port led to delivery of a sucrose pellet in the feeder port and an incorrect response led to a 5 s timeout. Mice were allowed 10 s to initiate a trial and 6 s to make a choice response. The SD stage consisted of 70 trials divided into seven 10-trial blocks; SD learning

criterion was nine correct responses within any 10-trial block, and if the subject did not attain criterion the SD stage was repeated on the next day. On the day after attaining SD criterion, the second stage, learned non-reward (LNR), was presented: the incorrect stimulus location at SD became the correct stimulus, the previously correct stimulus was removed and replaced by the blank panel, and the blank panel was replaced by a stimulus port and this became the new incorrect stimulus. Number of trials, learning criterion and times allowed for trial initiation and choice responding were all identical to those at the SD stage. At the LNR stage, the mouse must overcome non-reinforcement-avoid behaviour and acquire reinforcement-approach behaviour at the same stimulus, in the absence of the possibility to perseverate in responding at the previously correct stimulus (Nilsson et al., 2012). Measures of interest for SD and LNR were: omissions to initiate trials, omissions to emit choice responses, incorrect choice responses, total errors (i.e. the sum of the previous three error types)), and correct choice responses (CR). LNR testing began at day 16 and continued for 3-13 days depending on mouse-specific attainment of learning criteria for the SD and LNR stages. Testing was conducted 5 days/week. CSD mice were maintained in the same cage and next to the same CD1 mouse during this period. CSD sessions with maximum attack duration of 30 s were conducted on the other 2 days/week.

Statistical analysis

Statistical analysis of CSD effects was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA). In most cases an unpaired *t*-test or analysis of variance (ANOVA) was conducted. Where appropriate, ANOVA *post hoc* testing was conducted using the Bonferroni procedure. Analysis of covariance (ANCOVA) was used in Expt 8 (FR1 test). Statistical significance was set at $p \leq 0.05$. Where an estimate of variance is given this is the standard deviation (SD).

Results

CSD validation

We have previously shown that CSD leads to increased mean percent day-to-day body weight delta (Azzinnari et al., 2014). During the 15-day CSD, % daily body weight delta was calculated. For each experiment with *ad libitum* access to food (i.e. Expts 1-7, Fig. 1), CSD mice had higher day-to-day body weight delta than CON mice: for example, in Expt 1 (Fig. 1): CON 1.0 ± 0.3 %, CSD 2.1 ± 0.5 % ($p < 0.0001$). Given that one major aim was to investigate effects of psychosocial stress on the immune system, it is essential to minimise occurrence of skin wounds during CSD attacks. As reported previously, regular trimming of the lower incisor teeth of the CD-1 mice and timing the duration of daily physical attack to 1 min maximum, restricts wounding to surface abrasions and these occur rarely (Azzinnari et al., 2014). For example, in Expt. 1 (Fig. 1), during the 15-day CSD the median total number of surface skin wounds per mouse was 0 and the maximum was 3; there were no deep bite wounds throughout the study.

CSD induces peripheral and brain inflammatory responses

In the first experiment (Fig. 1), CSD effects on spleen and whole brain immune-cell status at day 16, were assessed. Spleens from CON and CSD mice were collected and splenocytes were analysed using flow cytometry. CSD decreased the number of lymphocytes ($CD45^+/CD11b^-$) ($t_{(10)}= 3.6$, $p < 0.005$, Fig. 2A, B), whereas the number of $CD45^+/CD11b^+$ myeloid cells was increased ($t_{(10)}= 2.4$, $p < 0.04$, Fig. 2A, C). In this latter population, identification of granulocytes and inflammatory monocytes was based on SSC-A and Ly6C staining (Fig. 2D). $SSC^{hi}/Ly6C^{int}$ granulocytes ($t_{(10)}= 2.4$, $p < 0.04$, Fig. 1E) and $SSC^{lo}/Ly6C^{hi}$ inflammatory monocytes ($t_{(10)}= 4.3$, $p < 0.002$, Fig. 1F) were both increased in the spleen

of CSD mice. Based on mean fluorescence intensity (MFI), the expression of TNF-α ($t_{(9)}$= 3.0, p < 0.02, Fig. 3A), IFN-γ ($t_{(9)}$= 4.0, p < 0.004, Fig. 3B) and Ki-67 ($t_{(9)}$= -3.0, p < 0.02, Fig. 3C) on myeloid cells was increased in CSD mice, while the surface expression of MHC II on myeloid cells was decreased in these same CSD mice compared to their CON counterparts ($t_{(10)}$= 3.6, p < 0.005, Fig. 3D).

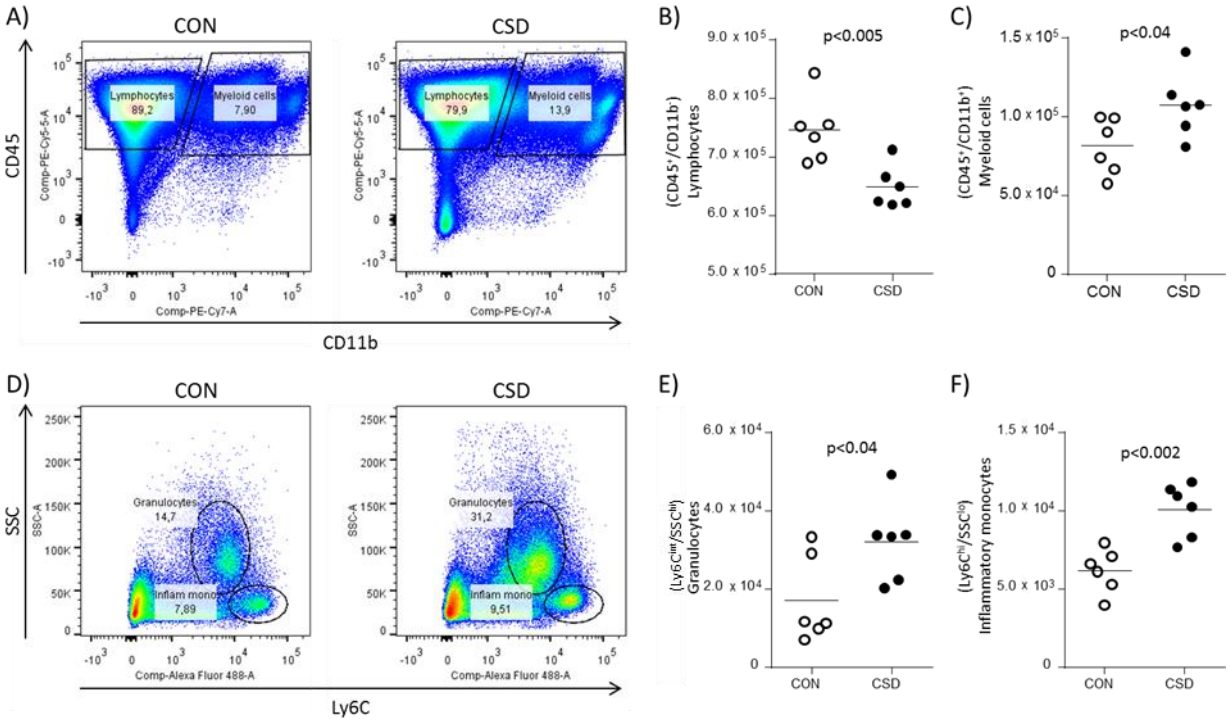

Figure 2. Effects of CSD on splenic leukocytes at day 16, as assessed using flow cytometry. Spleens were processed from 6 CSD and 6 CON mice. A) FACS dot plots for the percentage of total cells defined as lymphocytes and myeloid cells based on CD45/CD11b staining, in a representative CON mouse and CSD mouse. B) Cell counts for $CD45^+/CD11b^-$ lymphocytes in CON vs. CSD mice. C) Cell counts for $CD45^+/CD11b^+$ myeloid cells in CON vs. CSD mice. D) FACS dot plots for the percentage of total myeloid cells that were gated into different subsets, in a representative CON mouse and CSD mouse: $CD11b^+/SSC^{hi}/Ly6C^{int}$ = granulocyte, $CD11b^+/SSC^{lo}/Ly6C^{hi}$ = inflammatory monocyte. E) Cell counts for $Ly6C^+/SSC^{hi}$ granulocytes in CON vs. CSD mice. F) Cell counts for $Ly6C^{hi}/SSC^{lo}$ inflammatory monocytes in CON vs. CSD mice. p values were obtained in unpaired *t*-tests.

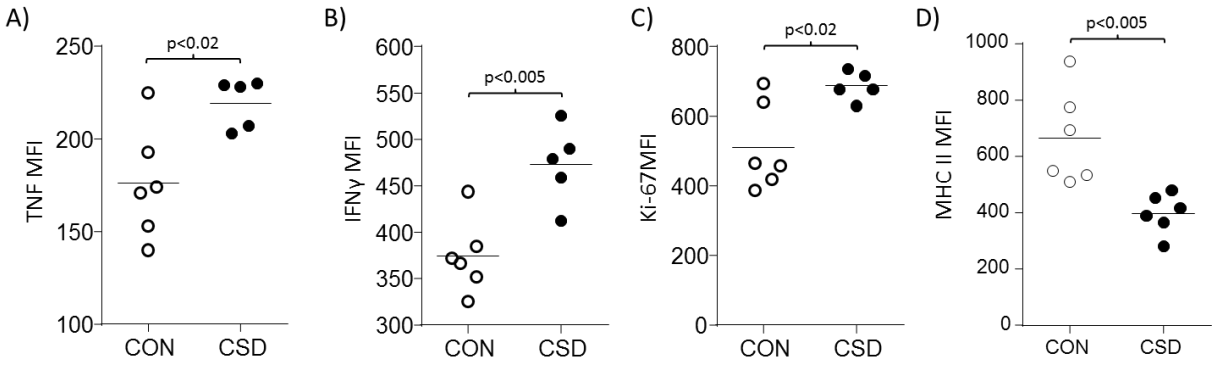

Figure 3. Effects of CSD on expression levels of activation and proliferation markers in $CD45^+/CD11b^+$ myeloid cells in the spleens obtained from 6 CON and 6 CSD mice at day 16 (same mice as in Fig. 2), as assessed using flow cytometry and the geometric mean of fluorescence intensity (MFI). A) TNF-α. B) IFN-γ. C) Ki-67. D) MHC II. p values were obtained in unpaired *t*-tests. In A), B) and C), the data point for one CSD mouse is missing because of a staining issue.

As stated above, CSD resulted in a decrease in the number of lymphocytes in the spleen. With respect to some specific T-cell types, CSD had no effect on the number of cytotoxic $CD8^+$ T cells ($p = 0.73$) or on the number of total $CD4^+$ T cells ($p = 0.28$) (Fig. 4A-C). Within $CD4^+$ T cells, there was no effect of CSD on counts for $CD4^+$/IFN-γ^+ Th1 cells ($p = 0.51$) or $CD4^+$/$Foxp3^+$T-reg cells ($p = 0.85$) (Fig. S1). However, CSD led to an increase in the number of $CD4^+$/$IL17A^+$ Th17 cells ($t_{(9)} = 3.1$, $p < 0.02$) (Fig. 4D, E).

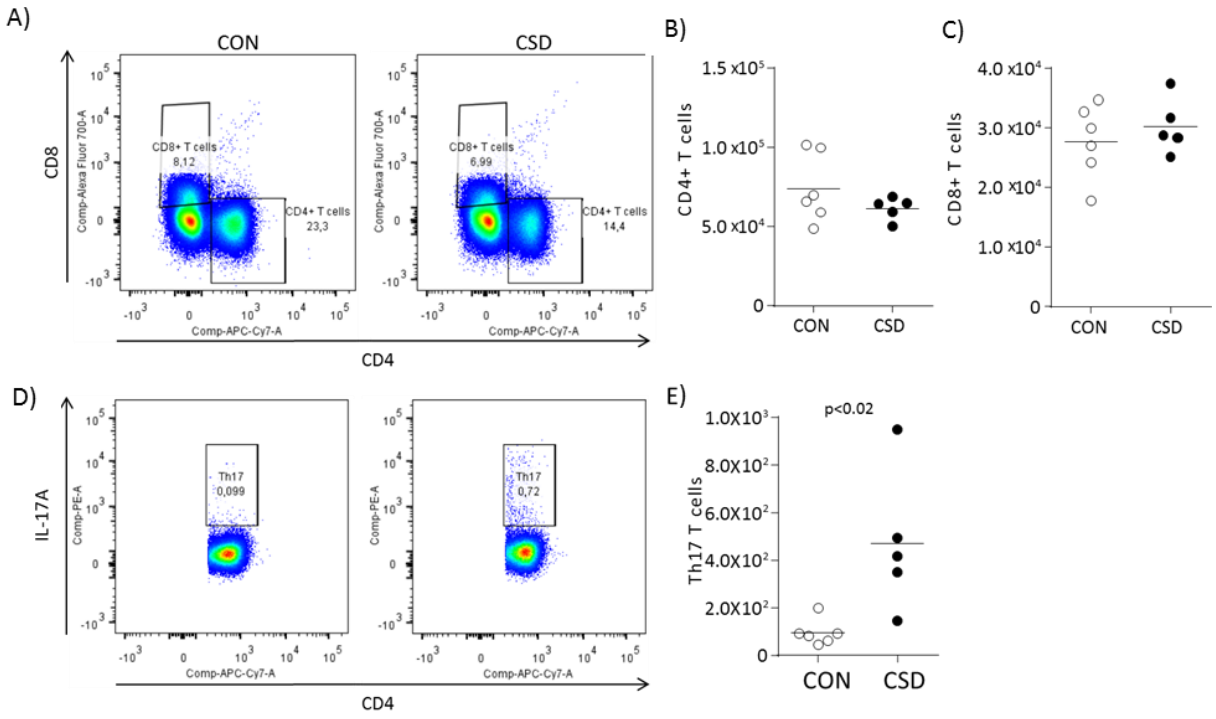

Figure 4. Effects of CSD on splenic T cells at day 16, as assessed using flow cytometry. Spleens were obtained from 6 CSD and 6 CON mice (same mice as in Fig. 2 and Fig. 3). A) FACS dot plots for the percentage of $CD4^+$ and $CD8^+$ T cells in a representative CON mouse and CSD mouse. B) Cell counts for CD4+ T cells in CON vs. CSD mice. C) Cell counts for CD8+ T cells in CON vs. CSD mice. D) FACS dot plots for the percentage of $CD4^+$ and $CD8^+$ T cells in a representative CON mouse and CSD mouse. E) Cell counts for Th17 T cells. The p value was obtained in an unpaired *t*-tests. In B), C) and E), the data point for one CSD mouse is absent because of a staining issue.

In a separate experiment (Expt 2, Fig. 1), the effects of CSD on the expression of genes encoding enzymes of the KYN pathway (Fig. 5A) in the liver, one of the major organs of KYN-pathway activity, were investigated using qPCR. The liver from CSD mice exhibited increased levels of *Ido2*, *Tdo2*, *Kmo*, *Kynu*, and *3-Haao*, with no significant increase in *Tdo1* (Fig. 5B-G). Liver expression of *Ido1* was barely detectable in CON and CSD mice (i.e. Ct > 36-37), in accordance with previous reports (Dai and Zhu, 2010). Given that activated myeloid cells and an activated KYN pathway are both associated with increased oxidative stress, we measured plasma levels of iNOS in these same mice. Plasma levels of iNOS were indeed increased in CSD compared to CON mice ($t_{(10)} = 2.6$, $p < 0.03$) (Fig. 5H).

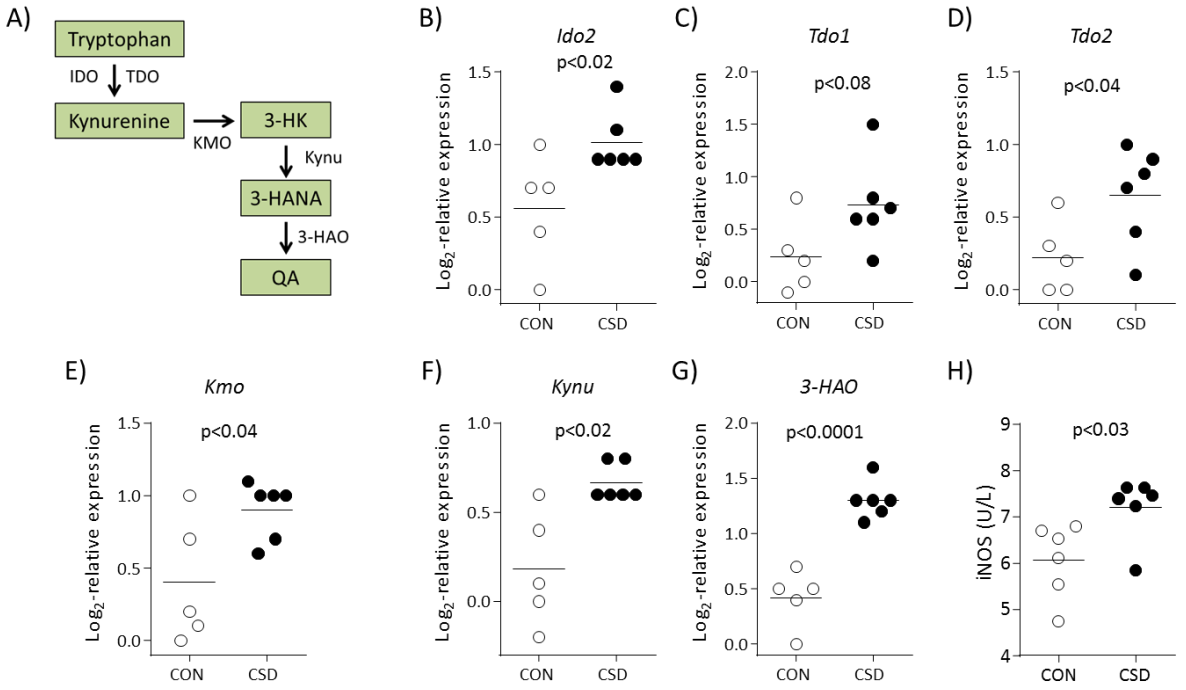

Figure 5. Effects of CSD on liver kynurenine-pathway enzyme gene expression and plasma iNOS levels at day 16. Livers and blood plasma were obtained from 6 CSD and 6 CON mice; liver data from one CON mouse are excluded because of unreliable RNA quantification. A) The metabolic steps in the kynurenine (KYN) pathway for which the enzymes studied are responsible. The KYN pathway is initiated by metabolism of tryptophan to N-formylkynurenine by indoleamine 2,3-dioxygenase 1 and 2 (IDO1, IDO2) and tryptophan 2,3-dioxygenase 1 and 2 (TDO1, TDO2); N-formylkynurenine is degraded by formamidase to kynurenine (KYN). KYN conversion to 3-hydroxykynurenine (3-HK) is catalysed by kynurenine 3-monooxygenase (KMO), and 3-HK to 3-hydroxyanthranilic acid (3-HANA) by kynureninase (KYNU). 3-hydroxyanthranilic acid 3,4-dioxygenase (3-HAO) converts 3-HANA to 2-amino-3-carboxymuconic-6-semialdehyde, which then rearranges to form quinolinic acid (QA) (Schwarcz et al., 2012). Liver mRNA expression levels of the genes for each of these enzymes was measured using qPCR: B) *Ido2*, C) *Tdo1*, D) *Tdo2*, E) *Kmo*, F) *Kynu*, G) 3-*HAO*, H) Plasma iNOS concentration measured using ELISA. p values were obtained in unpaired *t*-tests.

Moving to the analysis of CSD effects on immune status of the brain, in the same mice used to study spleen leukocytes (Expt 1, Fig. 1), we also isolated and analysed whole-brain mononuclear cells using flow cytometry. There was no CSD effect on the total numbers of brain lymphocytes ($CD11b^-/CD45^+$) ($p = 0.13$, Fig. 6A, B), macrophages ($CD11b^+/CD45^{hi}$) ($p = 0.43$, Fig. 6A, C), or microglia ($CD11b^+/CD45^{low}$) ($p = 0.55$, Fig. 6A, D). To assess brain region-specific effects of CSD on microglia, we conducted a separate experiment (Expt 3, Fig. 1) with the aim of measuring the microglia marker Iba1, in perfused brain sections using immunohistochemistry (IHC). In the VTA specifically, CSD led to an increase in the area that was Iba1-immunoreactive (IR) ($t_{(13)} = -2.8$, $p < 0.02$, Fig. 6E, F). This effect was specific to the VTA, with there being no CSD effect on this measure in nucleus accumbens ($p = 0.83$), medial prefrontal cortex ($p = 0.23$) or amygdala ($p = 0.45$) (data not shown). In a separate cohort of mice (Expt 4, Fig. 1), the VTA was punched and mRNA levels of another marker of microglia activation, *Cd11b*, were measured using qPCR; CSD mice exhibited increased *Cd11b* expression relative to CON mice ($t_{(20)} = -2.7$, $p < 0.02$, Fig. 6G).

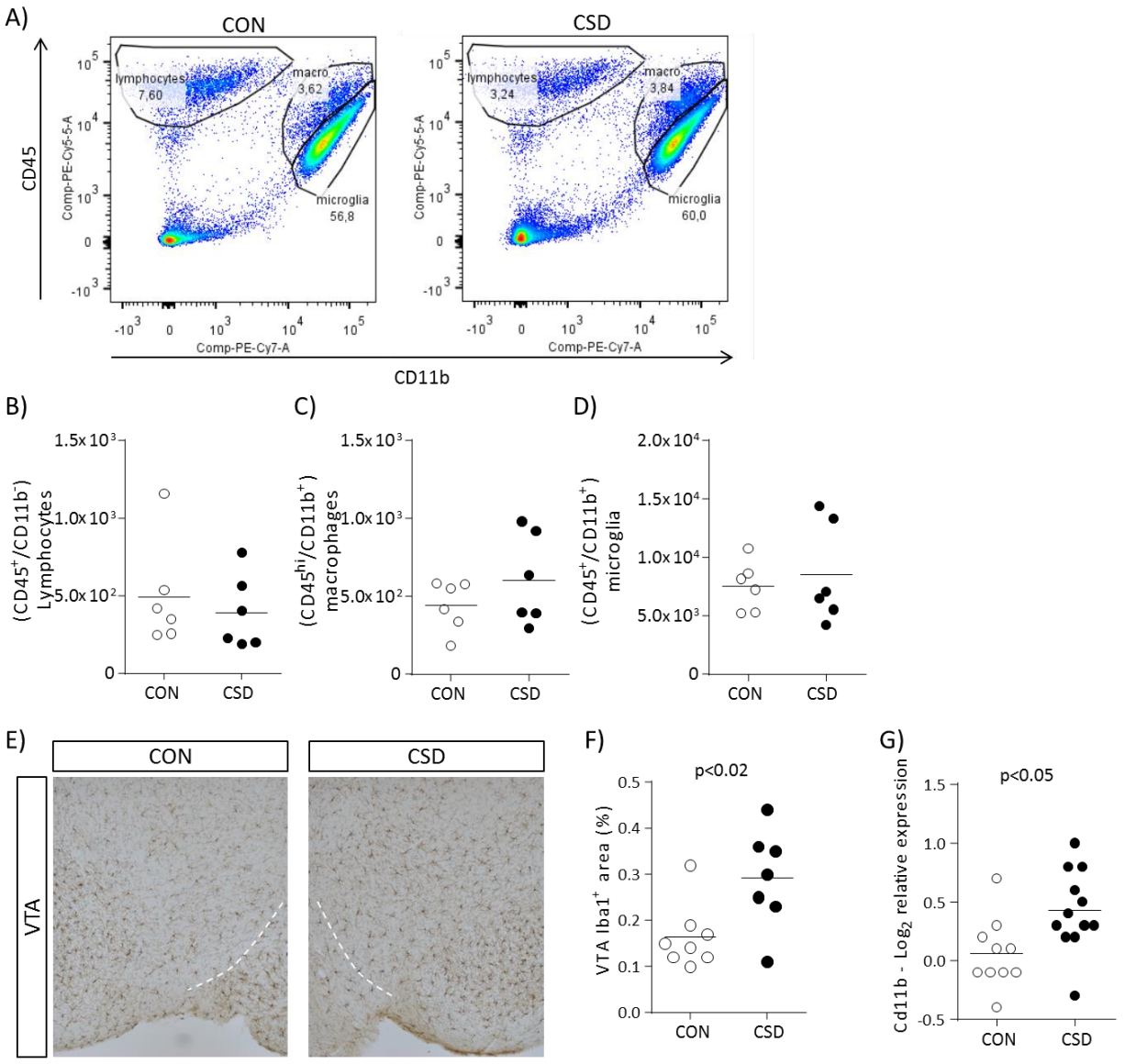

Figure 6. Effects of CSD on brain measures of immune status at day 16. Data are derived from three separate experiments. A)-D). Fresh brains were obtained from 6 CSD and 6 CON mice (same mice as in Fig. 2-4): A) FACS dot plots for whole-brain mononuclear cells showing the percentage of lymphocytes ($CD11b^-/CD45^+$), macrophages ($CD11b^+/CD45^{hi}$) and microglia ($CD11b^+/CD45^{lo}$) in a representative CON mouse and CSD mouse. Cell counts for: B) Lymphocytes. (C) Macrophages. (D) Microglia. E)-F) Brains from 7 CSD and 8 CON mice were perfused for Iba1 immunohistochemistry: E) Representative images of $Iba1^+$ staining in VTA from a CON mouse and a CSD mouse. F) Percentage area exhibiting $Iba1^+$ staining in the VTA. G) Fresh brains were obtained from 12 CSD and 10 CON mice: qPCR mRNA expression of *CD11b* mRNA in the VTA. p values were obtained in unpaired *t*-tests.

CSD induces changes in the mesolimbic dopamine system

In the same mice that exhibited increased expression of *Cd11b* in VTA, we also measured VTA (Fig. 7A) expression of several genes essential to DA function, namely tyrosine hydroxylase (Th), vesicular monoamine transporter 2 (*Vmat2*), dopamine transporter (*Dat*) and dopamine receptor 2 (*Drd2*) (Expt 4, Fig. 1). There was no effect of CSD on expression of *Th* ($p = 0.14$, Fig. 7B), CSD led to an increase in *Vmat2* ($t_{(20)} = -2.1$, $p < 0.05$, Fig. 7C), CSD was without effect on *Dat* ($p = 0.29$, Fig. 7D) and on Drd2 ($p = 0.12$, Fig. 7E). In a separate cohort of mice (Expt 5, Fig. 1), the NAcc was investigated in terms of tissue levels of DA and its major metabolites DOPAC and HVA, using HPLC-ED. The ratio

DOPAC/DA, the major measure of DA turnover, was reduced in CSD relative to CON mice ($t_{(19)}$= 2.2, p < 0.05, Fig. 7G). There was no effect of CSD on the ratio HVA/DA (p = 0.57, Fig. 7H).

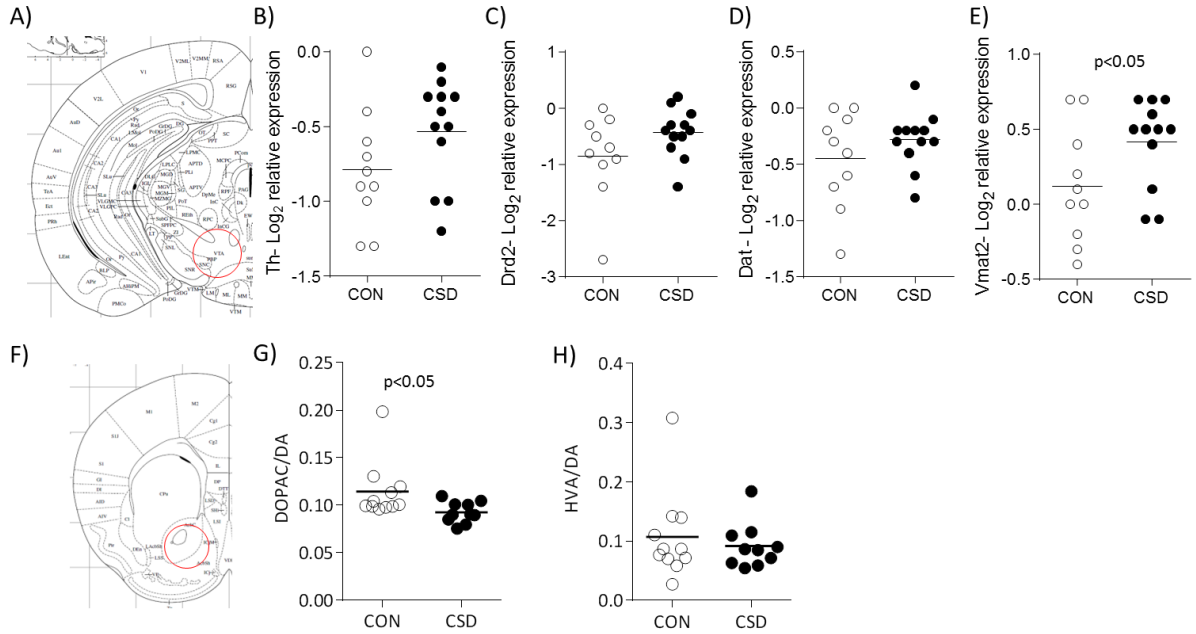

Figure 7. Effects of CSD on neural measures of the mesolimbic dopamine system at day 16. Fresh brains were obtained from 12 CSD and 10 CON mice (same mice as in Fig. 6G). A) Representative figure from (Franklin and Paxinos, 2008) depicting the VTA target region that was punched and processed for qPCR. Log2-relative mRNA expression for: B) Tyrosine hydroxylase, C) Vesicular monoamine transporter 2, D) Dopamine transporter, E) Dopamine receptor 2. F) Representative figure from (Franklin and Paxinos, 2008) depicting the NAcc target region that was punched and processed for HPLC-ED. G-H) Ratio of the metabolites DOPAC and HVA to DA as estimates of DA turnover in projections to the NAcc. p values were obtained in unpaired *t*-tests.

Building on the *ex vivo* evidence that CSD alters the state of the mesolimbic DA system, we used the DAT inhibitor GBR 12909 to investigate CSD effects both i*n vivo* and *ex vivo* (Expt 6, Fig. 1). On day 16, mice injected with GBR 12909 or vehicle were assessed in terms of locomotor activity during a 2 h period beginning 30 min after i.p. administration. In a 2 (Stress) x 2 (Drug) ANOVA of locomotor activity, there were main effects of Stress ($F_{(1, 21)}$=7.4, p<0.02) and Drug ($F_{(1, 21)}$=34.7, p < 0.0005) and a borderline non-significant Stress X Drug interaction ($F_{(1, 21)}$=3.7, p<0.07, Fig. 8A). *Post-hoc* analysis revealed similar (baseline) activity in CON-VEH and CSD-VEH mice, increased activity in both CON-GBR and CSD-GBR mice relative to their respective VEH groups, but decreased activity in CSD-GBR versus CON-GBR mice. Thereafter, mice were returned to their home cage and activity was measured from 13:00 h to 07:00 h the following day. In a 2 (Stress) x 2 (Drug) x 19 (Hour-block) ANOVA of activity scores, there was a Stress x Time interaction ($F_{(18, 378)}$=1.9, p < 0.05, Fig. 9). *Post hoc* analysis identified that CSD mice were less active that CON at the end of the active (dark) phase, i.e. at 16:00 to 20:00 h.

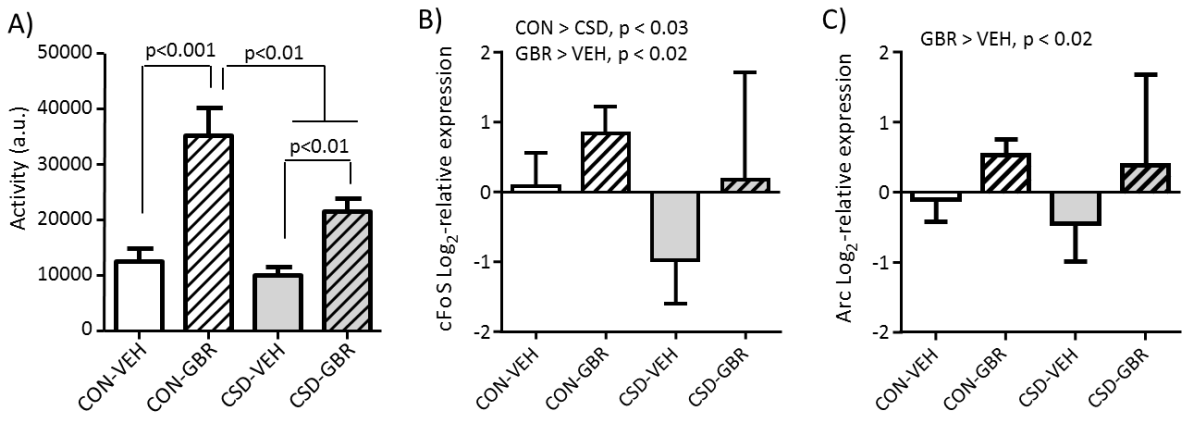

Figure 8. Effects of CSD and the specific DAT inhibitor GBR 12909 (6 mg/kg i.p.) on activity and accumbens immediate-early gene expression at days 16-17. CSD (N=12) and CON (N=13) mice were assigned equally to receive either GBR 12909 or saline vehicle (VEH). A) Day 16 locomotor activity in an arena during 2 h, starting 30 min after injection. B)-C) Day 17 nucleus accumbens immediate-early gene expression, with brains collected at 30 min after a second injection. B) *c-Fos,* C) *Arc.* Values are mean +/- SD. p values were obtained in 2-way ANOVA followed by *post hoc* Bonferroni test.

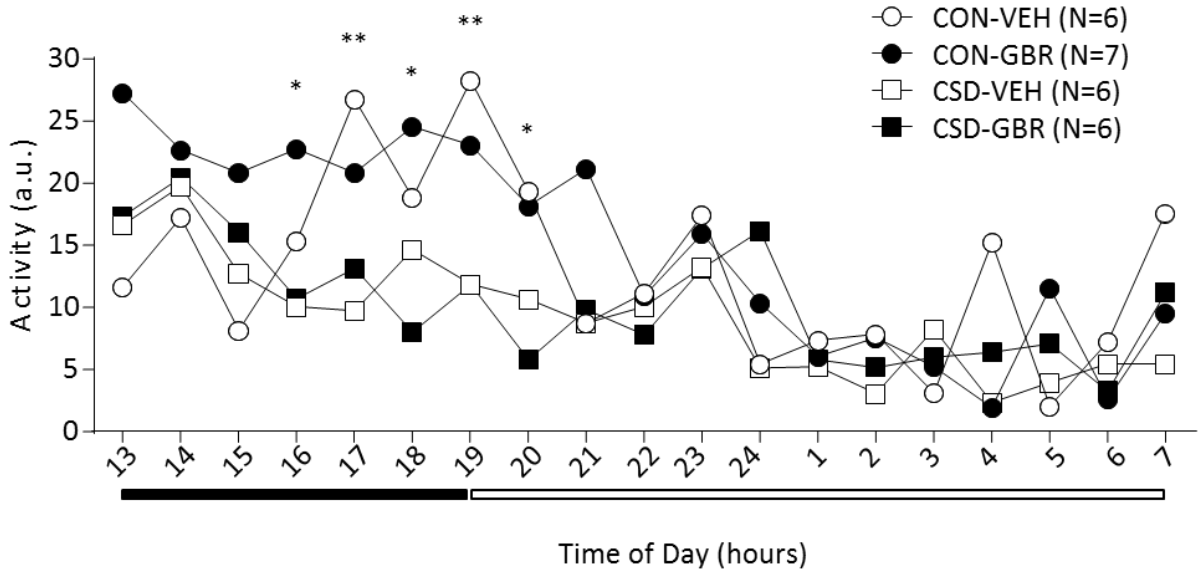

Figure 9. Effects of CSD and GBR 12909 on activity in the home cage at days 16-17 (same mice as in Fig. 8). Mice were returned to their home cage at 12:00 h following i.p. injection of GBR 12909 or VEH 2.5 h earlier, and activity was monitored across the remaining active (dark) phase and the subsequent inactive (light) phase. Activity was sampled at 1 min intervals, and average values for each hour were used for analysis. * $p < 0.05$, ** $p < 0.01$ for Stress X Time interaction in ANOVA followed by time-specific CSD vs. CON *post hoc* Bonferonni tests.

On day 17, mice received a second injection of the same drug and were killed after 30 min, and the brains collected for qPCR of the immediate-early genes *c-fos* and *Arc* in the NAcc. For *c-fos* expression (Fig. 8B) there were main effects of Stress ($F_{(1, 21)}=6.19$, $p < 0.03$) and Drug ($F_{(1, 21)}=7.44$, $p < 0.02$) and no interaction ($p = 0.57$). *c-fos* expression was reduced in CSD mice relative to CON mice and increased by GBR 12909 to a similar extent in CSD and CON mice. For A*rc* expression (Fig. 8C), there was a main effect of Drug ($F_{(1, 21)}=6.06$, $p < 0.02$), with GBR 12909 increasing *Arc* expression, in the absence of a Stress effect.

CSD induces deficits in operant responding for reward

To allow comparison with several previous studies that have assessed CSD effects in the two-bottle sucrose/saccharin test (see Discussion), this test was also included in the present study (Expt 7, Fig. 1). CSD mice (N=14) and CON mice (N=6 pairs) that had been exposed to the test conditions several times prior to CSD, were tested at day 16. There was no effect of CSD on absolute saccharin drinking (CON 5.2 ± 2.1 g, CSD 5.3 ± 1.1 g, $p = 0.87$). Interestingly, CSD mice exhibited increased water drinking (CON 0.08 ± 0.00 g, CSD 0.21 ± 0.02 g, $t_{(18)} = -2.35$, $p < 0.04$). Primarily due to the latter, CSD mice exhibited a borderline non-significant decrease in % saccharin preference (CON 98.1 ± 0.04 %, CSD 96.1 ± 0.04 %, $p < 0.07$).

In a separate, final experiment (Expt 8, Fig. 1), mice were trained in the operant paradigm for sucrose pellet reinforcement prior to CSD/CON. Operant training required mice to be maintained at 90-95% of baseline body weight (BW) and they were returned to 100% baseline BW prior to CSD/CON. During the 15-day CSD/CON procedure, CON mice were 100 ± 3 % baseline BW and CSD mice were 103 ± 3 % ($p < 0.01$). Normal diet was always fed several hours after operant testing and was always fully consumed within the dark phase, such that mice did not eat for ≥ 12 h prior to operant tests. To assess reward motivation at CSD day 8, mice were given the fixed-ratio 1 (FR1) test. Given the difference in % baseline BW, behavioural measures were analysed using ANCOVA with % baseline BW included as a covariate. Reduced motivation for sucrose pellets in CSD mice was evidenced by, compared to CON mice: an increase in the number of omissions to initiate trials ($F_{(1, 24)} = 5.70$, $p < 0.03$, Fig. 10A); a decrease in the number of pellets earned ($F_{(1, 24)} = 5.29$, $p < 0.04$, Fig. 10C); and an increase in the session duration ($F_{(1, 24)} = 7.55$, $p < 0.02$, Fig. 10D). There was no effect of CSD on omissions to emit stimulus responses ($F_{(1, 24)} = 0.18$, $p = 0.67$, Fig. 10B).

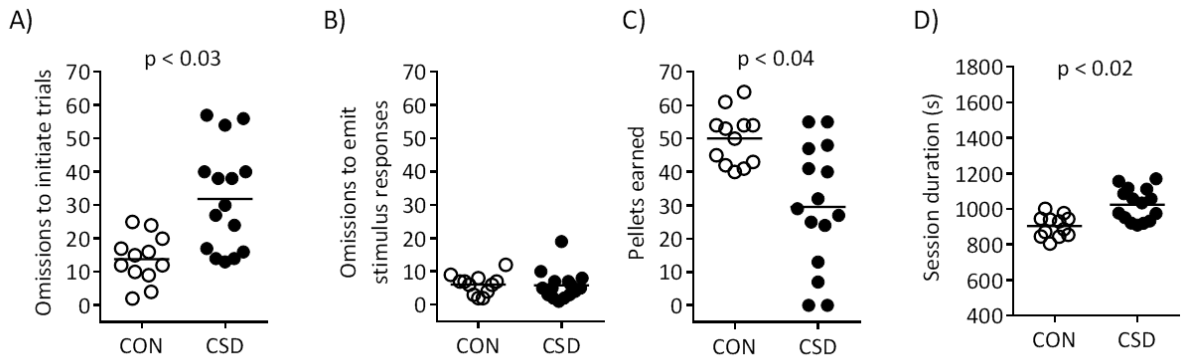

Figure 10. Effects of CSD on operant reward motivation at CSD day 8 in the FR1 test with a time limit of 10 s to initiate each trial and 6 s to emit an operant response. Mice were trained in the operant test and then allocated to CSD (N=15) or CON (N=12). A) Omissions to initiate trials. B) Operant responses in initiated trials. C) Pellets earned. D) Session duration. The bar represents the mean for each group per measure. p values were obtained using ANCOVA, and % baseline BW was included as a covariate.

Beginning on day 16, the same mice were investigated in the LNR test. Mice were maintained at 95% baseline BW: it was necessary to provide CSD mice with more normal diet (2.2±0.4 g/day) to maintain the target BW (actual BW: 95±2% baseline) than was required by CON mice (1.4±0.3 g/day) to maintain the target BW (actual BW: 97±4% baseline) (CSD vs. CON daily diet weight during LNR testing, $p < 0.001$). In the spatial discrimination (SD) stage, there was no effect of CSD on the measures omissions to initiate trials ($p = 0.53$), omissions to make choice responses ($p = 0.25$), incorrect choice responses ($p = 0.52$), total errors ($p = 0.62$), and correct choice responses ($p = 0.18$).

At the LNR stage, CSD mice exhibited a borderline non-significant increase in omissions to initiate trials ($t_{(14.4)}$= -2.4, p < 0.06, Fig. 11A), an increase in incorrect choice responses ($t_{(24)}$= -2.2, p < 0.04, Fig. 11C), and an increase in total errors ($t_{(17.6)}$= -2.4, p < 0.05, Fig. 11D). There was no effect of CSD on omissions to emit choice responses (p = 0.16, Fig. 11B) or on correct choice responses (p = 0.19).

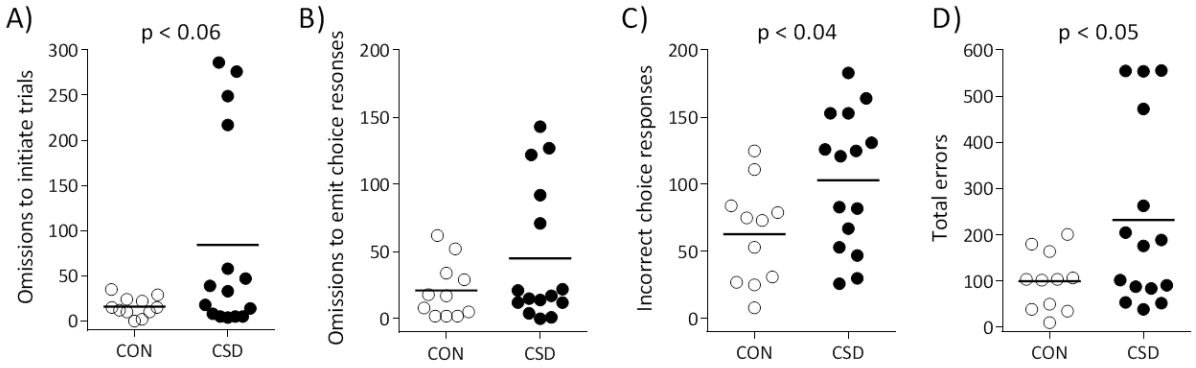

Figure 11. Effects of CSD on operant behaviour in the learned non-reward stage of the LNR test. Following simple discrimination testing to the learning criterion, mice were tested to learning criterion in the LNR test. One CON mouse had to be excluded due to a technical failure in the first LNR session (CON N=11, CSD N=15). A) Omissions to initiate trials. B) Omissions to make choice responses. C) Incorrect choice responses. D) Total errors. The bar represents the mean for each group per measure. p values were obtained in unpaired *t*-tests.

Discussion

The overall aim of the present study was to investigate effects of chronic exposure to a psychosocial stressor on peripheral and CNS immune responses, on the mesolimbic DA system and the associated changes in reward processing, in mice.

We have previously demonstrated that CSD induces peripheral and CNS immune system (Azzinnari et al., 2014), in terms of increased plasma levels of TNF and IL-6, splenomegaly and increased expression of genes associated with inflammatory pathways in the hippocampus, mPFC and amygdala (Azzinnari et al., 2014). Here we extend the evidence for and understanding of CSD effects on immune system status in periphery and brain. Splenocyte flow cytometry identified an increased number of $CD11b^{+}/CD45^{+}$ monocytes in CSD relative to CON mice, in terms of both $SSC^{hi}/Ly6C^{int}$ granulocytes and $SSC^{lo}/Ly6C^{hi}$ inflammatory monocytes. Using the manipulation of repeated social defeat (RSD) in mice, Avitsur et al. (2002) also demonstrated increased splenic granulocytes and $CD11b^{+}$ monocytes. The splenic myeloid cells of CSD mice displayed increased proliferation, as indicated by expression of the marker Ki67, and also increased expression of the pro-inflammatory cytokines TNF-α and IFN-γ. Thus, splenic myeloid cells might be a major contributor to the increased plasma levels of TNF-α and IFN-γ observed in CSD mice (Azzinnari et al., 2014; Fuertig et al., in prep). Interestingly, MHC II expression by splenic myeloid cells was reduced in CSD mice. Decreased monocyte MHC II surface expression combined with increased blood pro-inflammatory cytokines is associated with poor clinical outcome during the course of severe infections (Lekkou et al., 2004), and intracellular accumulation of MHC II molecules in myeloid cells occurs in severe infection (Fumeaux and Pugin, 2002). Further investigations are needed to assess whether intracellular sequestration of MHC II is also occurring in CSD mice.

Regarding the effects of CSD on the adaptive immune system, flow cytometry identified a reduced number of total splenic $CD11b^{-}/CD45^{+}$ lymphocytes in CSD mice. Given that this was not

attributable to splenic $CD4^+$ and $CD8^+$ T cells, the observed reduction in splenic lymphocytes might be due to a decrease in B cells, and future experiments will need to test for this. Despite the lack of effect on total CD4+ T cells, CSD mice did exhibit a specific increase in $CD4^+$/IL-$17A^+$ Th17 cells. Th17 cells selectively produce proinflammatory cytokines including IL-17A and F, IL-22, IL-6 and TNF-α (Kimura and Kishimoto, 2010). IL-17-producing T-helper cells play an important role in the induction of autoimmune diseases, including multiple sclerosis (MS) and an animal model thereof, experimental autoimmune encephalomyelitis (EAE) (Aranami and Yamamura, 2008). Naive T cells differentiate into Th17 cells under the influence of TGF-β, which is responsible for maturation of T regulatory (Treg) cells, and of IL-6 (Kimura and Kishimoto, 2010). As noted above, CSD mice exhibit increased blood IL-6 (Azzinnari et al., 2014), which might drive the development of Th17 cells and a consequent imbalance in Treg/Th17 ratio. Imbalance between Th17 and Treg cell numbers has been observed in mice exposed to chronic and acute stress (Beurel et al., 2013; Hong et al., 2013). Furthermore, administration of peripheral and CNS Th17 cells resulted in depression-relevant behaviour (Beurel et al., 2013). Schmidt et al. (2010) demonstrated increased T cell effector functions in the periphery of mice exposed to social stress. Once in the brain, Th17 cells can induce neuronal dysfunction in both cell bodies and axons (Siffrin et al., 2010).

Despite the marked activation effect of CSD psychosocial stress on splenic immune cells, there was no effect of CSD on whole brain numbers of $CD11b^+$/$CD45^{lo}$ microglia, $CD11b^+$/$CD45^{hi}$ macrophages or lymphocytes. Previous studies have provided clear evidence for microglia activation (Gerecke et al., 2013; Nair and Bonneau, 2006; Wohleb et al., 2012), and also increased monocyte trafficking to the brain (Wohleb et al., 2014a), due to various stressors, including repeated social defeat (RSD) and restraint. To investigate whether CSD leads to brain region-specific microglia activation we quantified Iba1 immuno-activity in VTA, NAcc, amygdala and mPFC. CSD led to increased Iba1 staining in the VTA specifically. Increased expression of *CD11b* in a separate cohort of CSD mice provided further support for microglia activation in the VTA. Interestingly, Tanaka et al. (2012) also showed microglia activation on the VTA of CSD mice, although this co-occurred with microglia activation in other brain areas (e.g. mPFC). One possible explanation for the lack of CSD effect on microglia/macrophages at the whole-brain level and on brain regions other than VTA examined using IHC might reside in the highly dynamic response of microglia to stressful stimuli. Indeed, microglia activation was observed on day 4 specifically of a 6-day restraint stress procedure (Nair and Bonneau, 2006), and Kreisel et al. (2014) found microglia activation after 2 days but microglia decline after 4 weeks of chronic unpredictable stress. Thus, in future studies it will be important to assess CSD effects on microglia activation and monocyte/T cell at earlier time points. At the same time the roust evidence for increased activation of microglia in the VTA has important implications for DA function and depression-relevant behaviour.

Increased pro-inflammatory cytokine levels activate the KYN pathway (Campbell et al., 2014). For example, IDO and TDO expression is increased in mouse liver and brain after lipopolysaccharide administration (Walker et al., 2013), with increased TDO activity probably mediated indirectly via increased glucocorticoid receptor activation (Badawy, 2013). KYN pathway abnormalities have been reported in various human neurological (Schwarcz et al., 2012) and psychiatric disorders (Haroon et al., 2012; Myint and Kim, 2014), including depression (Fukuda, 2014; Muller and Schwarz, 2007; Steiner et al., 2012; Zunszain et al., 2011). A reduction in the kynurenic acid (KA): quinolinic acid (QA) ratio has been reported for depressed patients relative to control probands (Savitz et al., 2015c). Moreover, QA levels are altered in brain tissue in depression: microglial QA expression was increased in the cingulate cortex, whereas it was decreased or unchanged in the hippocampus (Busse et al., 2015; Steiner et al., 2008). Here we demonstrate that CSD leads to activation of the KYN pathway in

liver, as indicated by increased mRNA levels for the key enzymes *Ido2*, *Tdo2*, *Kmo*, *Kynu*, and *3-Haao* that catalyse the pathway of conversion of tryptophan to QA. Also in rats, restraint stress led to increased liver TDO activity and KYN levels, and co-administration of the TDO inhibitor allopurinol attenuated the depression-relevant behavioural changes observed in these rats (Gibney et al., 2014). Chronic mild stress in mice induced activation of KYN pathway enzymes in skeletal muscle and liver and increased plasma KYN and brain 3-HK levels (Agudelo et al., 2014). The present evidence complements our findings that CSD induces increased KYN and 3-HK in plasma and brain, and that these and increased conditioned fear expression are reversed by repeated administration of an IDO1 inhibitor (Fuertig et al., in prep).

Further to the above lines of evidence that CSD led to a state of immune-inflammatory activation in the day following the chronic procedure, plasma levels of inducible nitric oxide synthase (iNOS) were also increased in CSD mice, the same cohort that exhibited upregulated gene expression for KYN-pathway enzymes. Immune activation induces the expression of iNOS, with pro-inflammatory cytokines (Sheng et al., 2011) and kynurenines (Colin-Gonzalez et al., 2013) being known activators iNOS and of oxidative stress. iNOS is one of the synthases responsible for catalysing nitric oxide (NO) production and the subsequent formation of free radicals (Miller et al., 2009b). Oxidative stress has been proposed to participate in depression pathophysiology (Bakunina et al., 2015; Miller et al., 2009a), and increased oxidative stress markers have been reported in the blood (Bakunina et al., 2015; Lopresti et al., 2014) and brain (Michel et al., 2010; Shelton et al., 2011) of depressed patients. Interestingly, chronic stress-induced depression-like behaviour in rats can be prevented by administration of iNOS inhibitors (Wang et al., 2008). Oxidative stress is known to be important in the pathophysiology of neurodegenerative diseases, including Parkinson's disease (PD) for example (Miller et al., 2009b), and to act at DA neurons among others (Bove et al., 2005).

To assess the effects of CSD on the mesolimbic DA system we measured levels of DA and its metabolites, DOPAC and HVA, in NAcc tissue. Absolute NAcc tissue levels of DA, DOPAC and HVA were not affected by CSD. Nonetheless, CSD mice exhibited decreased DA turnover as assessed by the (reduced) DOPAC/DA ratio. Tanaka et al. (2012) report an increase in the DOPAC/DA ratio in NAcc and mPFC in mice after a single CSD exposure and a decrease in the same ratio after 10 days of CSD, when comparing 10-day to 1-day values within CSD subjects, but not relative to 10-day CON mice. We did not observe a CSD effect on DA turnover in the mPFC (data not shown). It is important to note that there are a number of methodological differences between the standard CSD procedure used in the Tanaka et al. study (Golden et al., 2011) and our procedure (Azzinnari et al., 2014), which aims to minimize physical wounding, and this could account for between-study differences. Reduced DA turnover in the basal ganglia has been reported for depression (Bowden et al., 1997). Regarding the expression of DA-related genes in the VTA, there was CSD effect of on expression levels of *Drd2, Th* or *Dat*, and *Vmat2* expression was increased in the VTA of CSD mice. Several studies have reported association of reduced VMAT2 expression/function with depression-relevant behaviour; for example mutant *Vmat2* +/- mice display a depression-like phenotype (Fukui et al., 2007), administration of the VMAT2 inhibitor tetrabenazine reduces reward motivation in mice (Nunes et al., 2013a), and exposure of rats to repeated swim stress leads to down-regulation of VMAT2 in NAcc and dorsal striatum. Nonetheless, elevated platelet VMAT2 density has been observed in depressed subjects (Zucker et al., 2002). There is high similarity between the pharmacodynamic characteristics of platelet and brain VMAT2 (Zucker et al., 2001), and increased platelet VMAT2 density has been proposed to reflect a compensatory enhancement of the capacity to accumulate monoamines in the vesicles in the presence of reduced monoamine turnover, as is proposed to occur in depression (Zucker et al., 2002). Interestingly, Duchemin et al. (2009) showed enhanced expression of VMAT2

mRNA and protein in midbrain DA neurons in a mouse model of nicotine abstinence, which was coincident with decreased basal DA release. Thus, the increased *Vmat2* expression observed in the VTA in CSD mice might represent a compensatory mechanism to overcome a monoaminergic deficit.

To assess effects of CSD on DA function, the selective DA transporter blocker and therefore reuptake inhibitor GBR 12909 was administered acutely as a DAergic probe. In mice, administration of GBR 12909 increases extracellular DA levels in the NAcc and dorsal striatum (Abdallah et al., 2009) and induces a hyper-locomotor response (Irifune et al., 1995). Whilst CSD and CON mice did not differ in their basal locomotor activity in a familiar arena, CSD mice exhibited attenuated locomotor responsiveness to GBR 12909 relative to that of CON mice. Several mechanisms might account for this CSD-induced hyposensitivity to GBR 12909. Firstly, it might be due to higher expression and therefore DA binding capacity of DAT in CSD mice. In rats, social stress increases DAT binding in the mPFC (Novick et al., 2011) and attenuates GBR 12909-induced extracellular DA accumulation (Novick et al., 2015). Although there was no CSD effect on *Dat* mRNA levels in the VTA in the present study, DAT protein levels in the NAcc, dorsal striatum and other regions should be investigated in CSD and CON mice. Second, it might be due to alteration of the post-synaptic signalling elicited by DA in striatal neurons. Striatal function is highly regulated by dopamine- and cAMP-regulated phosphoprotein (DARPP-32), a 32 kDa phosphoprotein that is enriched in medium spiny neurons in NAcc and dorsal striatum (Svenningsson et al., 2004), and that integrates the physiological effects of multiple extracellular and intracellular signals (Svenningsson et al., 2004). Interestingly, mutation of the Thr-34 (protein kinase A) site in DARPP-32 reduces the locomotor activating effects of cocaine, indicating that DARPP-32 phosphorylation at Thr-34 influences behavioural effects of DA transmission (Zachariou et al., 2006). Given that DARPP-32 phosphorylation is affected by CSD (Jin et al., 2015) and Darpp-32 gene expression is impacted by CSD (Azzinnari et al., 2014), CSD effects on DARPP-32 might mediate its attenuating effects on sensitivity to GBR 12909. Psychostimulants (e.g. cocaine, GBR 12909) acutely up-regulates the expression of the immediate early genes (IEGs) *c-fos*, *fosb* and A*rc* (Zachariou et al., 2006). In accordance with previous reports (Iadarola et al., 1993), we demonstrate here that GBR 12909 up-regulates the expression of the IEGs *c-fos* and A*rc* in the NAcc. We also demonstrate that CSD down-regulates *c-fos* expression. Given that the increase in *c-fos* expression induced by GBR 12909 was similar in CSD and CON mice, CSD-GBR 12909 mice exhibited lower average NAcc *c-fos* expression than CON-GBR 12909 mice, paralleling their lower locomotor response to the DA probe. CSD did not impact on NAcc *Arc* expression in the present study, whereas chronic mild stress did do so in mice (Agudelo et al., 2014). It has been shown in rats that induction of ΔFosB protein levels is reduced in the NAcc shell of individuals susceptible to chronic restraint stress (Poulin et al., 2014). ΔFosB is a truncated and unusually stable isoform of the immediate-early gene *fosb*, and it tends to accumulate overtime in the brain following chronic stimulation (Nestler, 2015). However, in mice, a CSD-induced increase in ΔFosB in the NAcc has been reported to mediate resilience to the behavioural effects of CSD (Vialou et al., 2010). Responsiveness of IEG expression to a novel stressor has been investigated following exposure to various chronic stressors: *c-fos* expression was reduced in several brains regions in mice exposed to either chronic subordinate stress (Stone et al., 2007), uncontrollable physical stressors (Ons et al., 2010; Ostrander et al., 2009), or chronic unpredictable mild stress (Raineki et al., 2014), indicating a reduced evoked neural function of brain regions involved in stress-reactivity and motivation (Stone et al., 2007).

In addition to their attenuated locomotor reactivity to a pharmacological DA probe, CSD mice also exhibited reduced activity in the home cage. Building on this evidence, we conducted a final experiment investigating CSD effects on behaviour in operant reward tests known to be sensitive indices of NAcc DA function (Bergamini et al., Submitted manuscript). Reduced interest in reward,

included impaired appetitive behaviour with respect to physical effort and cognitive flexibility required to obtain reward, is a core symptom and dimension of depression (i.e. DSM-5, RDoC) (Eshel and Roiser, 2010). To investigate for CSD effects on reward processing we deployed a fixed-ratio 1 schedule for reinforcement (FR1) with time constraints to respond and learned non-reward (LNR) test which requires relearning that the stimulus-non-reward association is now a stimulus-reward association and concomitant overcoming of avoidance due to learned non-reward (LNR). Critically, we have demonstrated elsewhere that these processes are impaired by depletion of NAcc DA (Bergamini et al., Submitted manuscript). In the FR1 test, conducted at CSD day 8, CSD mice were less motivated to obtain sucrose pellets than CON mice, particularly because of frequent omissions to initiate trials. Mice were tested at 100% baseline body weight, with the aim of maximising the palatable incentive-motivational value of sucrose pellets relative to their calorific value. In the LNR test, CSD mice were not deficient in the simple discrimination stage of the test, indicating that their motivation for reward (they were at 95% baseline body weight) and their stimulus-outcome association learning were intact. However, they demonstrated increased LNR, primarily due to increases in omission to initiate trials and commission of incorrect responses. Strikingly, these same two deficits, coincident with intact simple discrimination, also underlay increased LNR sensitivity in mice that had undergone depletion of NAcc DA (Bergamini et al., Submitted manuscript). In depression, the intra-/extra-dimensional set-shifting task has been used to assess learned non-reward (and perseveration), and patients exhibit increased LNR relative to healthy subjects (Michopoulos et al., 2006). Using a progressive ratio schedule of reinforcement we obtained further evidence that CSD decreases reward motivation, and using reversal tests we obtained evidence that it impairs reward-related cognitive processing (present thesis, Study C: Bergamini et al. (in prep-a)). In contrast to the approach of investigating CSD effects on operant behaviour for reinforcement taken here, previous studies of CSD effects on reward-directed behaviour have used the two-bottle test of reward consumption. Several studies have reported a decrease in sweet-solution preference relative to water (e.g. (Covington et al., 2009; Krishnan et al., 2007). Despite the clear evidence for decreased motivation for reward under effortful conditions in the FR1 test, CSD mice did not exhibit a clear decrease in saccharin preference in the present study. Furthermore, the tendency for a reduced saccharin preference was due to the increase in water consumption by CSD mice relative to CON mice. This latter effect can probably be attributed to the increase in feeding observed in CSD mice (present thesis, Study C: (Bergamini et al., in prep-a)). Unfortunately, studies that report on CSD-induced decreased sweet-solution preference do not include data on absolute consumption of sweet-solution and water (e.g. (Covington et al., 2009; Krishnan et al., 2007). Therefore, to summarise our findings for reward-directed behaviour, CSD led to decreased motivation for reward under effortful and ambiguous conditions but was without effect on direct reward consumption. Each of these findings corresponds to those reported for experimental studies of reward processing in depression: thus, patients are less likely to exhibit high effort to obtain reward (Sherdell et al., 2012) but do not show impaired consummatory pleasure as measured by the Sweet taste test (Dichter et al., 2010; Treadway, 2015; Treadway and Zald, 2011).

This study provides some of the most robust evidence to-date in support of the hypothesis that stress-induced peripheral and brain inflammation co-occur with attenuated mesolimbic DA function including decreased interest in reward.

Supplementary Information

Table S1. List of primer sequences used in this study

Gene	Primer F (5'->3')	Primer R (5'->3')
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC
Hprt1	CTTCCTCCTCAGACCGCTTT	TTTTCCAAATCCTCGGCATA
Tbp	CATCTCAGCAACCCACACAG	GGGGTCATAGGAGTCATTGG
Th	AGAAGAGCCGTCTCAGAGCA	GGGCATCCTCGATGAGACT
Slc18a2 (Vmat2)	CTCCTCACCAACCCATTCAT	AAGGCATAGCTGCTGGAGAA
Slc6a3 (Dat)	TGATGCACATAGCAGCAACTC	AGGTCATCAATGCCACGACT
Drd2	GACACCACTCAAGGGCAACT	TCCATTCTCCGCCTGTTCAC
c-fos	TCCTACTACCATTCCCCAGC	TGGCACTAGAGACGGACAGA
Arc	TGAAGCAGCAGACCTGACAT	GTGTGAGGACTCAGCCCCT
Itgam (Cd11b)	ATTCGGTGATCCCTTGGATT	GTTTGTTGAAGGCATTTCCC
Ido1	CAAAGCAATCCCCACTGTATCC	ACAAAGTCACGCATCCTCTTAAA
Ido2	CCTCATCCCTCCTTCCTTTC	GGAGCAATTGCCTGGTATGT
Tdo1	AACATGCTCAAGGTGATAGCTC	GAACCGAGAACTGCTGTACCA
Tdo2	AGGAACATGCTCAAGGTGATAGC	CTGTAGACTCTGGAAGCCTGAT
Kmo	GGGGAAAAGAGTGGCTGTTA	CCACGCGAATATCTTCCCTA
Haao (*3-HAO*)	GGAGGCCCCAATACCAGGA	TATAGGCACGTCCCGGTGTT
Kynu	TCAAACCCTCCCATTTTGTTGG	CCCCTTGTTTTCGGTGTTATCTT

Abbreviations: Actb, actin, beta; Hprt1, hypoxanthine phosphoribosyltransferase 1; Tbp, TATA box binding protein; Th, tyrosine hydroxylase; Slc18a2, solute carrier family 18 (vesicular monoamine), member 2; *Slc6a3,* solute carrier family 6 (neurotransmitter transporter, dopamine), member 3; Drd2, dopamine receptor D2; *c-fos*, FBJ osteosarcoma oncogene; Arc, activity regulated cytoskeletal-associated protein; Itgam, integrin alpha M; Ido1, 2, indoleamine 2,3-dioxygenase 1,2; Tdo1,2, tryptophan 2,3-dioxygenase 1,2; Kmo, kynurenine 3-monooxygenase; Haao, 3-hydroxyanthranilate 3,4-dioxygenase; Kynu, kynureninase.

Figure S1

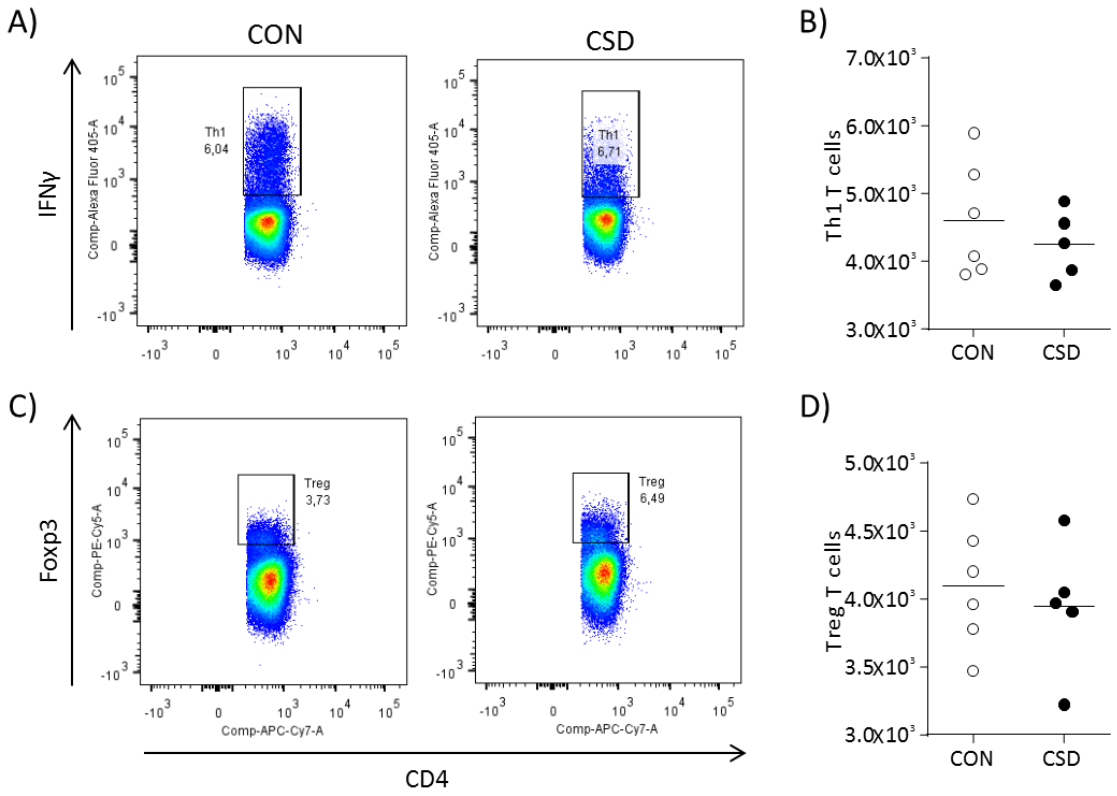

Figure S1. Effects of CSD on splenic T cells. Mice were subjected to CSD for 15 days or to CON conditions, and flow cytometric analysis was performed 24 h after the last CSD attack. Cells counts for $CD4^+/IFN\gamma^+$ Th1 cells (A, B), and $CD4^+/Foxp3^+$ Treg cells (C, D).

Study C

Mouse chronic social stress disrupts reward motivation and cognitive flexibility and certain of these effects are responsive to the antidepressant agomelatine

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* Giorgio Bergamini designed the study, carried out the experiments and data analysis, and wrote the manuscript

Abstract

Depression is a major and heterogeneous neuropsychiatric disorder, one core symptom of which is diminished interest in activities consistent with attenuated motivation to engage with positive situations and stimuli. Agomelatine (AGO) is a novel antidepressant with serotonergic 2C (5-HT_{2C}) receptor antagonism and melatonergic (MT1/2) receptor agonism properties. AGO is efficacious in increasing interest in positive activities in depressed patients, as assessed by specific questionnaire. Translational automated operant tests with positive reinforcement can also be applied in humans, and indeed animal models, to study reward processing and the aetio-pathophysiology underlying deficits thereof. Here we present three iterative studies in mice with the overall aims of investigating effects of chronic psychosocial stress on appetitive reward processing and whether AGO reverses these. In a first study, non-manipulated mice were trained on a complex reversal learning (CRL) test, and acute AGO (25 mg/kg p.o.) and a 5-HT_{2C} antagonist both increased reversals completed relative to vehicle (VEH). In a second study, mice were exposed to chronic social defeat (CSD) or control handling (CON) on days 1-15, and AGO or VEH on days 10-22: In a motivation test on day 15, CSD mice were less motivated and there was a tendency for AGO to reverse this effect. In a CRL test on day 22, CSD mice were less accurate and completed fewer reversals; repeated AGO (25 mg/kg p.o.) reduced the CSD effect on reversals, associated with increased and adaptive perseveration. In a third study, mice with continuous operant access to water and saccharin solution in the home cage were exposed to CSD or CON on days 1-15: CSD mice developed decreased appetitive-operant responding for saccharin and water during the active circadian period, decreased saccharin drinking during this period, and increased water drinking during the inactive period. In a separate cohort of CSD mice, repeated AGO (25 mg/kg p.o.) was without effect on these stress-induced changes. Therefore, mouse CSD led to depression-relevant reduced appetitive interest in reward under discrete-test and home-cage conditions, and repeated AGO reduced specific deficits observed under demanding discrete-test conditions.

Introduction

Depression is a major and heterogeneous neuropsychiatric disorder, with core psychopathological symptoms of depressed mood, diminished interest in activities, and increased fatigue, together with a number of common symptoms including weight loss or gain, psychomotor agitation or retardation, insomnia or hypersomnia, and reduced concentration and attention (DSM-5, 2013; ICD-10, 1994). The recent Research Domain Criteria (RDoC) project (Cuthbert, 2015; Cuthbert and Insel, 2013) proposes a classification system that takes diagnostic systems into account but also identifies the importance of understanding individual psychopathologies, which have been organised into domains: for example, the Positive valence systems domain, which includes approach motivation, responsiveness to reward and reward learning; and the Arousal/modulatory systems domain which includes arousal, biological rhythms and sleep-wake (Cuthbert and Insel, 2013). Integrating RDoC with depression symptoms, the Positive valence systems domain is clearly relevant to diminished interest in activities, and the Arousal/modulatory systems domain is clearly relevant to insomnia or hypersomnia. Depression is not well-understood in terms of aetio-pathophysiology, and patients with different symptom combinations are also likely to differ at the pathophysiological level. The RDoC approach is conducive with translational research aimed at increasing understanding of disorder pathophysiology. This includes evidence obtained in animal models that combine manipulations with aetiological validity with behavioural readout tests with face validity for specific psychopathologies (Pryce and Seifritz, 2011).

Chronic psychosocial stressors are major aetiological risk factors for depression (Kendler et al., 2003; Kessler, 1997). In mice, one manipulation that has been proposed to model aspects of this human environmental factor is chronic social defeat (CSD), which comprises 10-15 days of continuous intruder status in the home cages of different dominant mice but protected by a divider with brief daily experience of actual physical attack and defeat (Golden et al., 2011; Kudryavtseva et al., 1991). Mouse CSD has been reported to result in decreased preference for gustatory reward, namely sweet-tasting sucrose solution, over water in the two-bottle test of consummatory behaviour (Krishnan et al., 2007). Interestingly, for human depression, it has been reported that patients do not show reduced consummatory pleasure when given a rewarding stimulus (Dichter et al., 2010). However, when patients are required to exhibit high effort to obtain reward, they are less motivated to do so (Sherdell et al., 2012). Such evidence that, in depression, positive-valence processing is reduced at the motivational level rather than the consummatory level, highlights the need for animal models of stress-induced impairment of reward motivation and anticipation. These processes can be best-studied using behaviour-outcome (operant) tests. To our knowledge there is currently no published report on the effects of CSD on operant reward-directed behaviour in mice.

In order to investigate reward motivation per se, a single operant stimulus can be used with reinforcement earned according to a specific schedule. In animal studies the reinforcer typically takes the form of palatable food. The progressive ratio schedule (PRS) test requires the subject to make an increasing number of responses to obtain successive rewards and is therefore sensitive to assessing the motivation for effortful responding for reward. By conducting the PRS test with the animal subject close to hunger satiety, the anticipated palatability of the sucrose-pellet reward becomes more important relative to its calorific value, thereby increasing the test's sensitivity for assessing reward motivation. For example, in adult rats it has been demonstrated that early life stress leads to decreased responding for reward in a PRS test (Leventopoulos et al., 2009). Using tests comprising two operant stimuli that need to be discriminated between in order to obtain reinforcement, then a

number of further depression-relevant reward processes can be investigated. When depression patients are assessed in discrimination tests that require low effort to obtain reward, typically either symbolic (emoticon) or monetary, they do not differ from healthy subjects in terms of accuracy of responding (Taylor Tavares et al., 2008). Their responding is characterised by high sensitivity to error feedback, however: when patients make an error and therefore fail to receive reinforcement on a trial, they are more likely to make an incorrect decision on the subsequent trial i.e. catastrophizing (Elliott et al., 1996; Elliott et al., 1997). The probabilistic reversal learning (PRL) test assesses reward-directed decision making under conditions of accurate and misleading reinforcement/feedback (Chamberlain, 2006; Cools et al., 2002; Evers et al., 2005; Jocham et al., 2009). The test comprises reversal learning, which requires responding to regular shifts in the contingencies - reward, non-reward - between the two operant stimuli and reinforcement and, superimposed on this, at a certain probability correct responses are not rewarded whereas incorrect responses are rewarded. The proportion of non-rewarded correct responses on which the subject shifts (to the incorrect stimulus) on the next trial, gives a measure of negative feedback sensitivity (NFS). High NFS is indicative of under-estimation of reward probability and is increased in depressed patients (Taylor Tavares et al., 2008). Recently, rodent automated operant PRL tests have been developed, firstly for rat (Bari et al., 2010) and then in our laboratory for mouse (Ineichen et al., 2012). In order to ensure that motivation is sufficient for rodents to engage in this demanding test for sucrose-pellet reinforcement, some food deprivation is essential. In the mouse PRL test, referred to here as a complex reversal learning (CRL) test, misleading feedback is restricted to non-rewarded correct responses. Non-manipulated mice exhibit: (1) reward-stay behaviour consistent with accurate monitoring of the average reward probability at each stimulus; (2) low perseveration and high NFS consistent with accurate monitoring of expected and unexpected non-reward; (3) reduced reversals achieved relative to a simple reversal learning test without non-rewarded correct responses (Ineichen et al., 2012).

Agomelatine (AGO; S 20098; *N*(2-(7-methoxy-1-naphthyl)ethyl)acetamide)) is a potent agonist at the melatonergic ($MT_{1/2}$) receptors as well as an antagonist at the serotonin 2C (5-HT_{2C}) receptor, and is a recently approved antidepressant (de Bodinat et al., 2010; Kennedy et al., 2014). During its preclinical development, using repeated dosing, AGO was efficacious in several rat and mouse models relevant to depression domains (de Bodinat et al., 2010). This included reversal of decreased saccharin consumption induced by chronic mild stress (Papp et al., 2003), reversal of specific learned helplessness (Bertaina-Anglade et al., 2006), antagonism of increased anxiety, poor physical state and decreased grooming induced by repeated corticosterone (Rainer et al., 2012), and antagonism of increased anxiety, immobility in the forced swim test, and altered circadian rhythm of activity and sleep-wake pattern induced by prenatal restraint stress (Mairesse et al., 2013; Marrocco et al., 2014; Morley-Fletcher et al., 2011). In clinical trials, AGO exhibits increased therapeutic efficacy relative to placebo in moderate-severe depression (Kennedy et al., 2014). When the core symptom/domain of interest-pleasure was measured using a self-report questionnaire, AGO exhibited greater efficacy relative to venlafaxine (Di Giannantonio and Martinotti, 2012). The 5-HT_{2C} receptor has also been demonstrated to be relevant in reversal learning. For example, a 5-HT_{2C} antagonist improved reversal learning associated with decreased perseveration (Boulougouris et al., 2008). One neurochemical effect of AGO is to increased dopamine release in the frontal cortex (Millan, 2003); ant-/agonism of specific dopamine receptors in this brain region increased perseveration (Floresco et al., 2006).

The iterative aims of the present mouse study were to: (1) Investigate the effects of CSD, which we have already demonstrated to lead to increased sensitivity to and impaired coping with aversive stimuli (Azzinnari et al., 2014), on reward processing in a PRS test and simple and complex reversal learning tests, and on reward processing and circadian activity in a home cage equipped with operant access to water and sweet solution. (2) Investigate the effects of the antidepressant AGO in terms of reversing CSD-induced changes in reward processing in these various tests and contexts.

Materials and Methods

Animals

Experiments were conducted with adult male C57BL/6J mice bred in house, maintained in littermate pairs, and aged 10 weeks and weighing 26-28 g at study onset. Male CD-1 mice (Janvier, Le Genest-Saint-Isle, France) used for social stress were aged 8 months, weighed 40-45 g, were ex-breeders, and caged singly. Mice were maintained on a reversed light-dark cycle (light off 07:00-19:00 h). Standard home cages were type 2L and maintained in an individually-ventilated caging system, and one study was conducted using IntelliCages (see below). Both cage types contained sawdust, tissue bedding and a sleep igloo. Temperature was maintained at 22°C and humidity at 50-60%. The standard diet was Complete Pellet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water, both available continuously unless otherwise stated below. BL/6J mice were handled on 5 days at study onset. The studies were conducted under a permit (170/2012) for animal experimentation issued by the Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice studied and any unnecessary stress to those mice that were studied.

Experimental design

Three studies (A, B, C) were conducted, each with a different, naive cohort of mice (Figure 1). The first stage of each study was operant training for sucrose-pellet reinforcement (A, B) or saccharin-solution and water (C) reinforcement. In Study A (N=24 BL/6J mice), we investigated the effects of acute agomelatine (AGO), melatonin and a 5-HT_{2C} receptor antagonist on behaviour in a complex reversal learning (CRL) test in otherwise non-manipulated mice. In Study B (N=44 BL/6J mice), we investigated the effects of chronic social defeat (CSD, days 1-15, versus control handling (CON)) and AGO (days 10-22, versus vehicle (VEH)) on behaviour in a progressive ratio schedule (PRS) test (day 15), a simple reversal learning (SRL) test (day 19) and the CRL test (day 22). Study C comprised two experiments in IntelliCage: Firstly, we investigated the effects of CSD (day 1-15, versus CON) (N=20 BL/6J mice) on appetitive and consummatory behaviour towards water and saccharin solution during the dark- and light-phases of the circadian cycle. Second, in a new cohort of mice (N=16 BL/6J mice) the effects of AGO (days 7-20, versus VEH) on these same measures were studied in CSD mice (day 1-15).

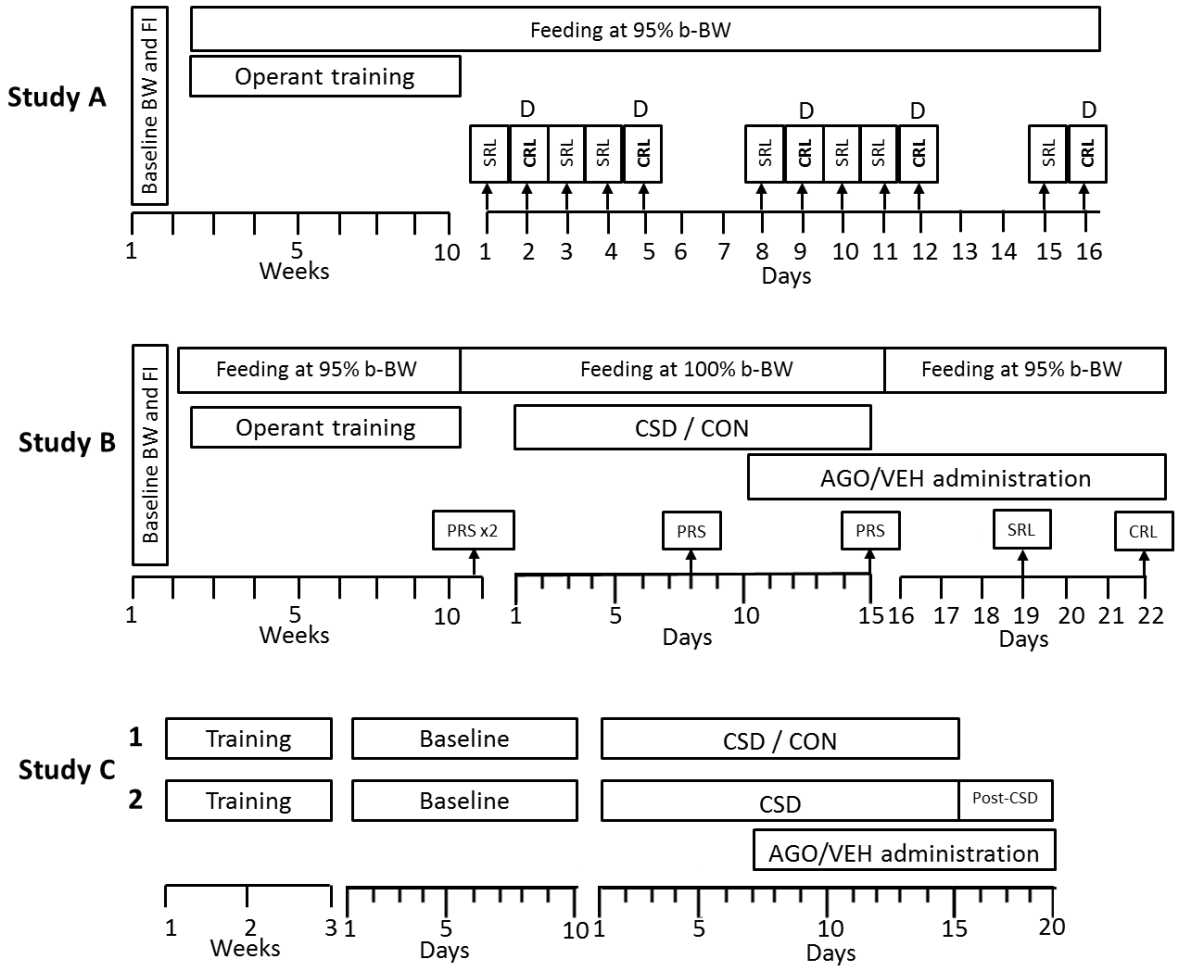

Figure 1. Experimental designs. Study A: effects of acute agomelatine melatonin and 5-HT_{2C} receptor antagonist on behaviour in a complex reversal learning test in otherwise non-manipulated mice. Study B: effects of chronic social defeat versus control handling and agomelatine versus vehicle on behavour in a progressive ratio schedule test and simple and complex reversal learning tests. Study C: Experiment 1, effects of CSD on appetitive and consummatory behaviour towards water and saccharin solution during the dark- and light-phases of the circadian cycle. Experiment 2, effects of agomelatine versus vehicle on these same measures. Baseline BW, b-BW: baseline body weight; FI: Food intake; SRL: simple reversal learning test; CRL: complex reversal learning test; D: acute drug; CSD: chronic social defeat; CON: control handling; PRS: progressive ratio schedule test; AGO: agomelatine; VEH: vehicle.

Chronic social defeat

Our refined protocol for chronic social defeat is described in detail elsewhere (Azzinnari et al., 2014). Briefly, in Study B, each BL/6 (CSD) mouse was placed singly in the 2L home cage of a CD-1 mouse, separated by a transparent, perforated divider. The CSD mouse was placed in the same compartment as the CD-1 mouse for either a cumulative total of 60-sec physical attack (chase, wrestle, bite) or 10 min maximum. To prevent bite wounds, the lower incisors of CD-1 mice were trimmed every third day across CSD. The CSD mouse x CD-1 mouse pairings were rotated so that CSD mice were placed in the home cage and confronted with a novel CD-1 mouse each day. This CSD procedure was conducted for 15 days, between 14:00-16:00 h. From day 16 until the end of operant testing, each CSD mouse remained in one cage with the same CD-1 mouse without further attacks. Control (CON) mice remained in littermate pairs, the standard condition in our laboratory, and were handled and weighed daily. In Study C, each IntelliCage was divided into two compartments by a transparent,

perforated divider, and a CSD mouse was placed in one compartment and a CD-1 mouse in the other. The CSD mouse was placed in the compartment occupied by the CD-1 mouse for either a cumulative total of 60-sec physical attack or 10 min maximum. The CSD mouse then remained in this compartment and the CD-1 mouse was transferred to the opposite compartment. CD-1 mice were rotated between IntelliCages every fifth day and lower incisors were trimmed every third day. CSD was conducted for 15 days, between 16:00-17:00 h. On days 16-20, the CSD and CD-1 mice were exchanged between compartments at 16:00 h without further attacks. Control littermate pairs of BL/6 mice were maintained singly in the same IntelliCage, one per compartment, and were exchanged between compartments daily at 16:00-17:00 h.

CSD effects on feeding homeostasis and appetite hormones

Given that the reversal-learning operant tests require food deprivation (Ineichen et al., 2012), that CSD is known to increase food intake and alter blood levels of appetite-regulating hormones in a pro-feeding direction (Kumar et al., 2013; Patterson et al., 2013), and that CSD effects on reversal learning tests were investigated in Study B, it was important to establish the effects of our CSD protocol on home-cage food intake and appetite hormone levels. In a separate experiment, daily intake of food pellet was measured in 11 littermate pairs for 5 days prior to allocation to CSD (N=12 mice) and CON (N=10 mice) and measurement of daily intake of food pellet for 15 days of CSD/CON. For mice in pairs, mouse pellet consumption per mouse was estimated by dividing the total amount of pellet eaten by two and adjusting for relative body weight. On day 16, mice were decapitated and trunk blood collected into 500 µl tubes coated with EDTA (Microvette, Sarstedt), and 4-2-aminoethyl-benzenesulfonyl fluoride (Sigma) was added at 1 mg/ml. The blood was centrifuged at 3000 rpm for 15 min at 4°C, the plasma was removed to Eppendorf Protein LoBind tubes, and acidified with HCl to a final concentration of 0.05 N. Plasma samples were stored at -25°C until determination of leptin and ghrelin using ELISA kits according to the manufacturer's protocol (Mouse Leptin EZML-82K, Rat/Mouse Ghrelin EZRGRA-90K, Merck Millipore).

Operant training and testing

Controlled feeding and body weight. In Studies A and B, body weight (BW) and food intake of BL/6 mice were measured every day for one week and the mean values were calculated and taken as baseline for each mouse. To ensure that mice were motivated for operant training, BW was reduced to 90-95% baseline by controlling daily pellet allowance. In Study B, at 5 days prior to and throughout CSD, mice were given sufficient daily pellet to return to and maintain baseline BW. They were then maintained at baseline BW for progressive ratio schedule testing and reduced to 95% baseline BW for reversal learning tests.

Operant boxes. Modular operant boxes (TSE Systems, Bad Homburg, Germany) were used for Studies A and B, details of which are provided elsewhere (Ineichen et al., 2012). Briefly, each box contained one (progressive ratio schedule test) or two (reversal learning tests) operant nose-poke port stimuli and one feeder that delivered one sucrose pellet per reinforcement (14 mg Dustless Precision Pellets, TSE Systems). Correct and incorrect nose-pokes and pellet retrieval were detected via infrared beam breaks. A loudspeaker above the feeder emitted a tone to signal reward delivery. After each session the boxes were wiped clean and odours were covered by wiping with 70% ethanol.

Training on fixed-ratio 1 (FR1) operant reinforcement. In Studies A and B, mice were conditioned to nose poke on a fixed-ratio 1 (FR1) schedule for sucrose pellet reinforcement during five sessions per week for 1-2 weeks, using the training stages detailed in (Ineichen et al., 2012). The basic settings were: trial onset was indicated by illumination of the nose-poke port(s); one nose poke (FR1) initiated tone and pellet delivery, and following pellet retrieval a 2.5 s inter-trial interval (ITI) was initiated; the session terminated after 40 reinforcements or 30 min.

Progressive ratio schedule (PRS) test. The PRS test was used in Study B, and conducted as described elsewhere (Ineichen et al., 2012). Briefly, only one nose-poke stimulus was used. The maximum session duration was 40 min. Session parameters were: required number of responses on first trial = 1, number of consecutive trials for which the ratio remained constant = 5, and number of responses by which the ratio increased per increment = 3, i.e. on trials 1-5, 1 response was required, on trials 6-10, 4 responses were required, on trials 11-15, 7 responses were required, and so on. If there was no single response within any 600-s period the break point was reached and the session terminated. Measures were total number of nosepokes, number of reinforcements attained, final ratio attained, and total number of responses at the feeder. In Study B, at 100% baseline BW, mice were given two PRS tests on consecutive days, and the scores on the second day were used to counter-balance allocation of mice to CSD and CON groups in terms of reward motivation. Mice were tested at 100% baseline BW for CSD effects in the PRS test at CSD days 8 and 15, with testing conducted at 09:00-12:00 h and therefore before the daily CSD session. Within CSD and CON mice, PRS behaviour at CSD day 8 was used to counter-balance allocation of mice to AGO and VEH drug groups.

Simple reversal learning (SRL) test. In Studies A and B, mice were trained on reversal learning, using two nose-poke stimuli positioned left and right of the central feeder. The final parameters for reversal training, and also the parameters used in the SRL test itself, were: On FR1, a response at the correct stimulus initiated tone and pellet delivery followed by 2.5 s ITI, and a response at the incorrect stimulus initiated 5-s time out. Consistent reward-stay behaviour in the form of eight consecutive correct responses/reinforcements was required for reversal. At reversal the previously incorrect stimulus was now correct and vice versa, so that nonreward-shift behaviour was required. The total number of pellets available was 48, giving a maximum of six reversals. Criteria for completion of reversal-test training were all 48 reinforcements obtained and consumed, a minimum of 18 ($p \geq 0.75$) reward-stay responses per stimulus, and a minimum of 3 reversals completed. Mice required 35-40 sessions, 5 days per week, from onset of operant training to reach this criterion for reversal learning. The final settings described above were also the settings for the SRL test. The measures of interest were as described below for the complex reversal test, except there was no measure, negative feedback sensitivity.

Complex reversal learning (CRL) test. The CRL test was used in Studies A and B, and was conducted as reported elsewhere (Ineichen et al., 2012) where the same test was described as probabilistic reversal learning. Briefly, the same parameters as in the SRL test were used with the addition of an overall probability of 0.15 that responses to the correct stimulus were not rewarded; these were called non-rewarded correct responses (NR-CRs). The first correct response per session was always rewarded and the maximum number of consecutive NR-CRs was two. A maximum of 60 pellets could be obtained and the maximum amount of reversals possible was 7, therefore. Measures of interest were: *Reward-stay probability*, the probability that the mouse stays at the active nose-poke stimulus after being rewarded on this stimulus, calculated as p (trials with response to the stimulus that was

correct on previous trial/total trials immediately following a correct trial). *Number of reversals completed*. *Errors (perseverations)/reversal*, immediately following reversal, the number of consecutive trials with non-rewarded responses to the previously correct but now incorrect stimulus/total reversal completed. *Trials/reversal*, mean number of trials required to complete a reversal. *Negative feedback sensitivity*, the probability that the mouse switches to the incorrect stimulus after receiving a NR-CR, calculated as p (trials with response to the opposite stimulus following a NR-CR/total trials immediately following a NR-CR). *Average latency to collect reward*, mean time (msec) required to collect the pellet from the feeder. *Session duration*, the time (sec) needed to finish the session by obtaining 60 pellets or 1800 sec maximum.

In Study A, all mice were given five CRL tests, each preceded by a different compound (see section Drugs below). Mice were given the SRL test on days between CRL tests, to ensure that they were exhibiting normal performance on the day prior to compound-CRL testing (Fig. 1).

In Study B, following CSD, mice were returned to food restriction on day 16, to reduce BW to 95% baseline. They were given a SRL test on day 19, and a CRL test on day 22; these were days 10 and 13 of daily AGO/VEH administration, respectively (Fig. 1, section Drugs below).

IntelliCage appetitive and consummatory behaviour. In Study C, IntelliCage (TSE) was used, a system for automated continuous monitoring of appetitive and consummatory behaviour of mice in their home cage, as described elsewhere (Cathomas et al., 2015a; Cathomas et al., 2015c). Briefly, each IntelliCage was divided at the centre to give two independent compartments, and placed in an attenuation chamber with a 12:12 h reversed dark-light cycle (light off 07:00-19:00 h). Mice were fitted with a subcutaneous transponder to record: visits to the operant devices located in each corner; operant nosepokes into a light sensor at the door in each corner that opened on FR1 to allow access to a drinking bottle; and the number of licks, measured by electrical contact of the tongue with the drinking tip. Littermate pairs were habituated to the cage for 3 days with operant doors open, and then the doors were closed so that mice had to nosepoke to open a door and access the water bottle for 20 sec, for a training period of 7 days. This was followed by 5 days of training with one water bottle and one saccharin solution (0.1%, sodium salt hydrate, Sigma) bottle, the corner locations of which were alternated each day at 17:00 h. For 10-day baseline data collection, the littermate pair was separated with one mouse placed in each compartment, and they were exchanged between compartments each day at 17:00 h. In the first experiment, this continued for a further 15 days in the case of CON mice. In CSD mice, one littermate was removed and replaced by a CD-1 mouse. The CSD procedure was conducted at 16:00-17.00 h for 15 days: the CSD mouse was placed in the compartment occupied by the CD-1 mouse for the physical attack, and then the CSD mouse remained in this compartment and the CD-1 mouse was transferred to the opposite compartment. CD-1 mice were provided with a normal water bottle. In the second experiment, after the 10-day baseline data collection, the CSD procedure was conducted on days 1-15 and thereafter mice were transferred between compartments without attack on days 16-20. AGO/VEH were administered at days 7-20. For analysis, four time periods of interest were identified: two during the light phase, 20:00-00:00, 00:00-04:00, and two during the dark phase, 08:00-12:00, 12:00-16:00. Therefore, the data obtained in the 3-4 hours directly after CSD and CON compartment transfer were not included in the data analysis.

Drugs

To establish dose and time effects on AGO levels, mice (N=6-7 per dose x time point) were orally (p.o.) administered 10 or 25 mg/kg AGO, and trunk blood and brain were collected after 1 h or 3 h

and stored at -80°C prior to analysis. AGO measurement in plasma and brain-tissue homogenate was performed according to an established method involving liquid-liquid extraction of a sample volume of 20 µl, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) detection. S 40706-1 (D5-20098) was used an internal standard. The limit of quantification (LLOQ) was 0.100 ng/ml (range 0.100 to 30 ng/ml). All LC-MS/MS measurements were performed by a central analytical laboratory.

All compounds were prepared in a vehicle of 1% hydroxyethylcellulose (HEC) in water. In Study A, acute AGO (S 20098) was studied at 10 and 25 mg/kg p.o., acute melatonin (MT) at 10 mg/kg p.o., and the 5-HT_{2C} antagonist S 32006 acute at 2.5 mg/kg p.o. A latin square design was used to test compound effects in a counterbalanced order across mice. A period of 3-4 days was allowed between each day of compound-CRL testing (Fig. 1). In Study B, 13-day repeated AGO was studied at 25 mg/kg p.o.. In Study C, 14-day repeated AGO was studied at 25 mg/kg p.o.. Compounds were administered at the end of the light phase (06:00-06:30 h) in all studies, and 1-2 hours prior to operant testing in Studies A and B.

Statistical analysis

Statistical analysis was conducted using SPSS (version 21, SPSS Inc., Chicago IL, USA). In Study A, compound effects on dependent measures in the CRL test were analysed using mixed model analysis of variance (ANOVA) with compound as a fixed effect and mouse as a random effect. In Study B, mixed model ANOVA with a 2 Stress (CSD, CON) x 2 Drug (AGO, VEH) design was used to study effects on measures in the PRS, SRL and CRL tests. CSD effects on food required to maintain baseline BW at specific time points were studied using *t*-tests. In Study C, in the first experiment a mixed model ANOVA with a 2 Stress (CSD, CON) x 4 Day-block (Baseline, CSD/CON 1-5, 6-10, 11-15) x 4 Time-block (20-00 h, 00-04 h, 08-12 h, 12-16 h) design was used to study effects on IntelliCage measures; and in the second experiment a mixed model ANOVA with a 2 Drug (AGO, VEH) x 5 Day-block (Baseline, CSD 1-5, CSD-Drug 6-10, CSD-Drug 11-15, Drug 16-20) x 4 Time-block (20-00 h, 00-04 h, 08-12 h, 12-16 h) design was used to study effects on IntelliCage measures. In each study, significant main effects or interactions were analysed using post hoc testing with Bonferroni correction in the case of multiple comparisons. Significance was set at $p \leq 0.05$ and a non-significant trend at $p < 0.10$. Data are given as means and where an estimate of variance is given this is the standard deviation (SD).

Results

Study A: Acute oral agomelatine enhances behaviour in the complex reversal learning test

Agomelatine blood and brain levels following p.o. administration at 10 or 25 mg/kg and sample collection after 1 or 3 h are given in Figure 2. In both plasma (Fig. 2A) and whole brain (Fig. 2B), mean AGO concentration was increased at 1 h after injection of 25 mg/kg p.o..

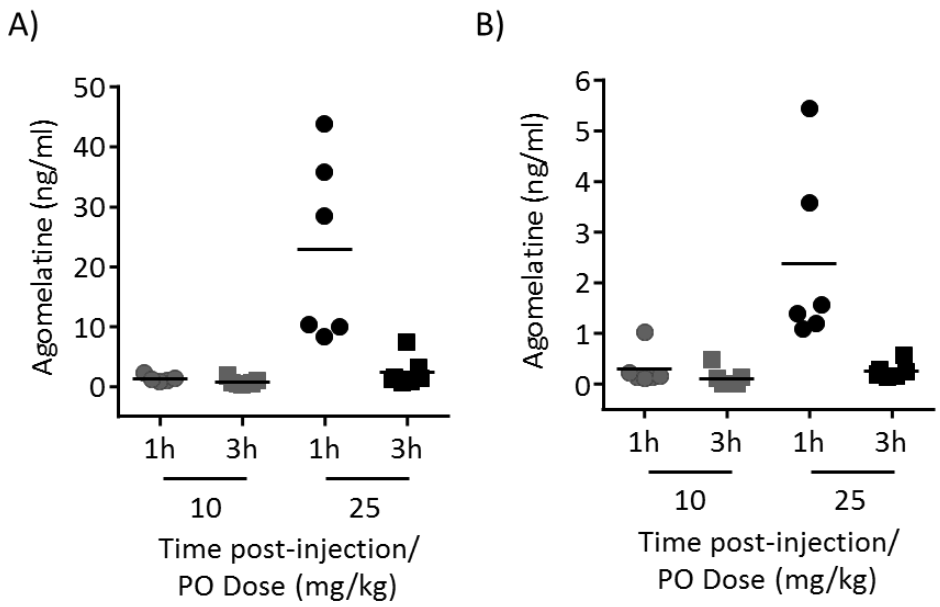

Figure 2. Agomelatine concentrations in (A) mouse trunk blood plasma and (B) whole brain tissue from the same mice, following oral administration of agomelatine at 10 or 25 mg/kg and sample collection after 1 or 3 hours. N=6-7 mice per dose and time point.

In the CRL test, each mouse was tested following acute p.o. AGO at 10 mg/kg (AGO 10) or 25 mg/kg (AGO 25), melatonin (MT) at 10 mg/kg and 5-HT_{2C} antagonist at 2.5 mg/kg (5-HT2C ANT), using a latin square design. As given in Figure 3, there was no Drug effect on reward-stay behaviour ($p=0.30$, Fig. 3A), and also no Drug effect on negative feedback sensitivity ($p=0.40$, Fig. 3B). For number of reversals completed, there was a main effect of Drug ($F(4, 114)=3.06$, $p<0.05$, Fig. 3C). Post hoc testing demonstrated that, relative to VEH, mice completed more reversals after AGO 25 ($p<0.05$) and 5-HT2C ANT ($p<0.03$).

Based on these pharmacokinetic and CRL test data, 25 mg/kg AGO was the dose selected for repeated oral administration in Studies B and C.

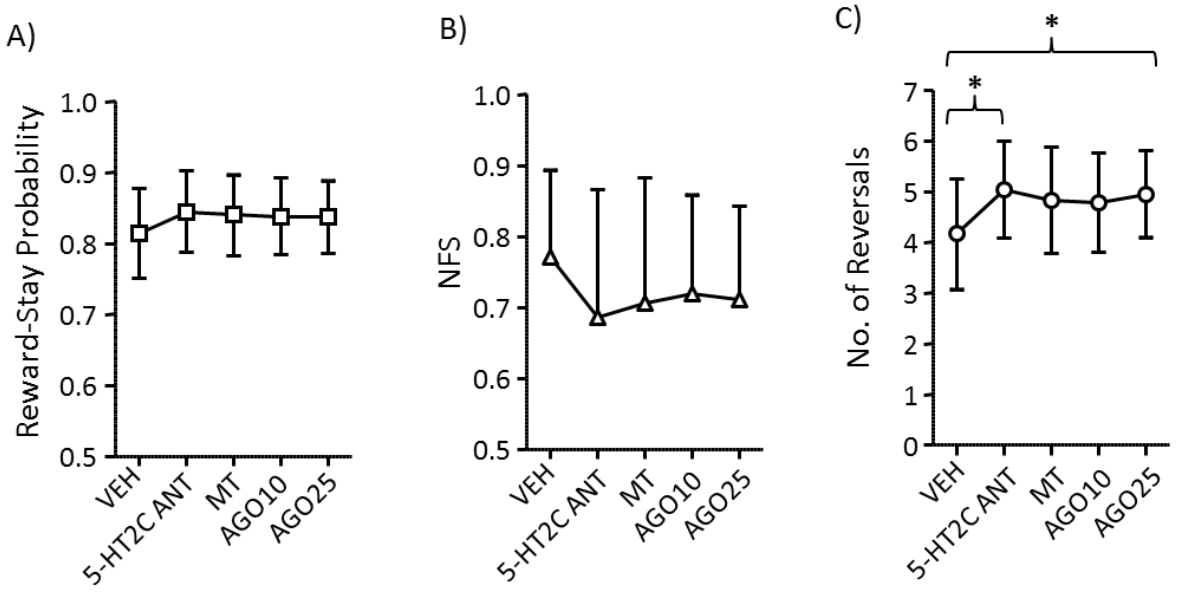

Figure 3. Study A: Compound- and dose-dependent effects of vehicle, agomelatine (AGO, 10 and 25 mg/kg),

melatonin (MT, 10 mg/kg) and 5-HT2C antagonist S 32006 (5-HT2C ANT, 2.5 mg/kg) on behaviour in the complex reversal learning (CRL) test. A latin square design for the order of compound/dose testing was used. Twenty four mice were studied, and each mouse obtained the maximum 60 reinforcements in each test session. Details of the experimental design are given in Figure 1. A) Probability of reward-stay responding. B) Negative feedback sensitivity with respect to switching stimulus after a non-rewarded correct response. C) Number of reversals. Values are mean±SD. * p<0.05 for post hoc Bonferroni pairwise comparisons conducted after significant effect of Drug in ANOVA.

Study B: CSD mice exhibit deficits in reward motivation and accuracy, and repeated agomelatine has some CSD-specific effects

CSD increases food intake and decreases plasma leptin levels

Prior to conducting Study B, in a separate cohort of mice we investigated CSD effects on food intake under ad libitum conditions and plasma levels of appetite hormones. CSD mice exhibited a marked increase in food intake: The mean daily weight of pellet eaten was calculated for the 5-day blocks, pre-CSD/CON -4 to 0, CSD/CON 1-5, CSD/CON 6-10, CSD/CON 11-15. There was a Group x Day-block interaction ($F(3,60)=9.98$, $p<0.0005$; Fig. 4A); pre-CSD/CON food intake was similar in CON and CSD mice, and CSD mice consumed more pellet than did CON mice at days 1-5, 6-10 and 11-15. For body weight, there was no effect of Group ($p\geq0.18$, Fig. 4B), and a main effect of Day-block ($F(3,60)=21.55$, $p<0.0005$) due to increase in BW over time. This increase in food intake in CSD mice co-occurred with a decrease in plasma levels of the appetite-suppressant hormone leptin in samples collected on day 16 (CON: 5.24±2.26 ng/ml, CSD: 2.12±0.93 ng/ml, $t_{(21)} = 4.77$, $p<0.0002$). There was no overall effect of CSD on plasma levels of the appetite-enhancing hormone ghrelin in the same mice (CON: 75.90±28.42 pg/ml, CSD: 107.80±70.24 pg/ml, $p=0.19$). These findings demonstrate the important need to control the BW and pellet allocation for each mouse individually and on a daily basis, in order to maintain all mice at the same BW relative to baseline, and therefore in as similar homeostatic state as possible, for operant testing.

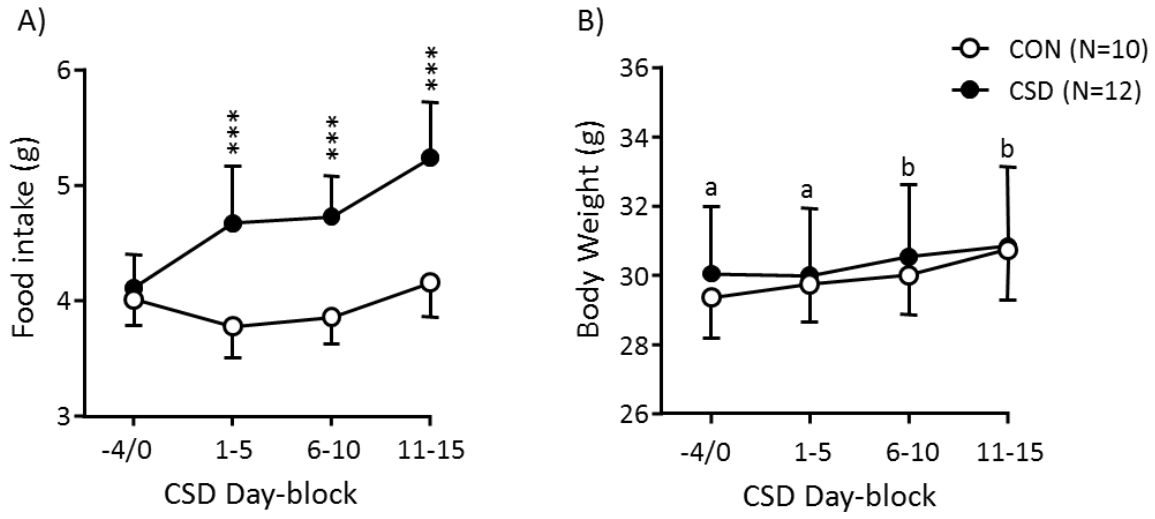

Figure 4. Effects of chronic social defeat on mouse food intake and body weight. A) Mean food intake per 5-day block. B) Body weight per 5-day block. The weight of food given and remaining was measured each day at 16:00 h. Values are mean+/-SD. In A) *** p<0.001 for post hoc Bonferroni pairwise comparisons conducted for Stress group in specific Day-blocks following Stress group x Day-block interaction in ANOVA. In B) day-blocks with different letters p<0.05 or less for post hoc Bonferroni pairwise comparisons following main effect of Day-block in ANOVA.

CSD and agomelatine effects in the progressive ratio schedule test

In Study B, with respect to baseline body weight and pellet intake, mice (N=22) that were later allocated to CON weighed 28.2±1.62 g (=100% baseline BW) and consumed 3.8±0.4 g pellet/day, and mice (N=22) that were later allocated to CSD weighed 28.7±1.6 g and consumed 3.8±0.5 g pellet/day. During the 15-day CSD/CON period, CON mice were given 3.1±0.5 g food pellet/day to maintain 100% baseline BW (actual values: 101.4±2.2%), whereas CSD mice had to be given 4.7±0.7 g food pellet/day to maintain 100% baseline BW (actual values: 101.5±1.5%). Therefore, as observed in the ad libitumg food intake experiment (Fig. 4), the CSD mice required more food to maintain physical homeostasis.

At CSD/CON day 8, which was prior to commencement of AGO administration (day 10, Fig. 1), mice were given a PRS test. At this stage there was no effect of CSD on the main measures of total number of responses (CON: 99±73, CSD: 104±113, p=0.85), reinforcements attained (CON: 17.2±5.6, CSD: 16.3±8.7, p=0.69), and final ratio attained (CON: 10.5±3.2, CSD: 9.6±5.2, p=0.46). The number of responses at the feeder was decreased in CSD mice (CON: 82±44, CSD: 55±34, $t_{(42)}$=2.33 p<0.03), suggesting decreased reward anticipation/motivation. Within CON and CSD groups, the total number of PRS responses was used to counter-balance allocation of mice to AGO and VEH groups.

At CSD/CON day 15, which was day 6 of AGO administration, a further PRS test was conducted. The data are presented in Figure 5. At this stage, there was a consistent effect of CSD on PRS test behaviour. Thus, for total number of responses there was a main effect of Stress group (F(1,40)=11.44, p<0.002, Fig 5A) with CSD mice making less responses. CSD mice also attained fewer reinforcements (p<0.001, Fig 5B) and a lower final ratio (p<0.001; Fig. 5C) relative to CON mice. The number of responses at the feeder was also decreased in CSD mice (CON: 298±156, CSD: 146±140, p<0.002). With the exception of three CSD-VEH mice which reached their break point, all mice continued responding to the end of the 40-min session. There was no Stress x Drug interaction or main effect of Drug for any PRS measure. However, as is evident in each data set in Figure 5, in CSD mice specifically there was an increase in the mean scores in mice receiving AGO; in *t*-tests, there was a borderline non-significant increase in the final ratio attained in CSD-AGO versus CSD-VEH mice (p=0.07; Fig. 5C).

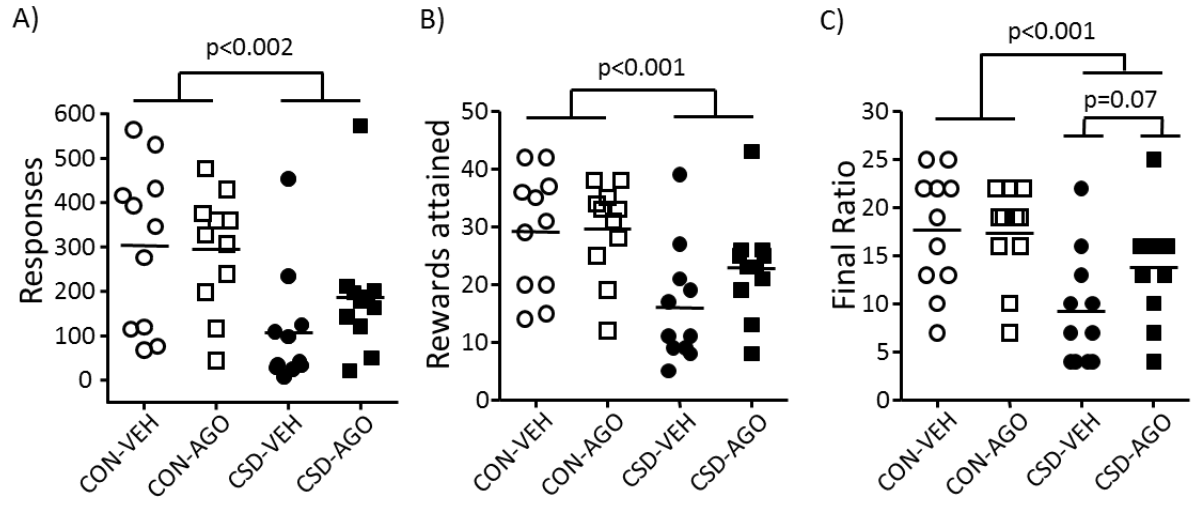

Figure 5. Study B: Effects of chronic social defeat and agomelatine (25 mg/kg per os) on mouse behaviour in the progressive ratio schedule test, at day 15 (last day) of CSD and after 6 days of AGO. The test was conducted 1-2 hours after daily administration of AGO/VEH at the end of the inactive phase. Details of the experimental

design are given in Figure 1. Eleven mice per group were tested, counter-balanced with respect to baseline behaviour in this test. (A) Total number of nosepoke responses. (B) Number of reinforcements attained. (C) Final ratio attained. In A-C, p values are for the main effect of Stress group in Stress group x Drug dose ANOVA. In C, in a Stress group-specific *t*-test, there was a borderline non-significant increase in the final ratio attained in CSD-AGO versus CSD-VEH mice.

CSD and agomelatine effects in the reversal learning tests

The same mice were then reduced to 95% baseline BW (CON: 96.2±2.2%, CSD: 97±3.3%, p=0.37) and studied in terms of CSD and AGO effects on reversal learning. At day 19, 4 days after the end of CSD and day 10 of AGO administration, mice were tested in the simple reversal test (Figure 6). There were main effects of Stress group, consistent with impaired test performance in CSD mice, for: number of reinforcements obtained (CON: 48±0, CSD: 40±13, p<0.007), probability of reward-stay responding (p<0.001, Fig. 6A), reversals completed (p<0.001, Fig. 6B), trials per reversal (p<0.02, Fig. 6D), average latency to collect reward (p<0.05, Fig. 6E) and session duration (p<0.03, Fig. 6F). There was no effect of CSD on perseverations per reversal (p=0.27, Fig. 6C). There was no Stress X Drug interaction or main effect of Drug for any SRL measure.

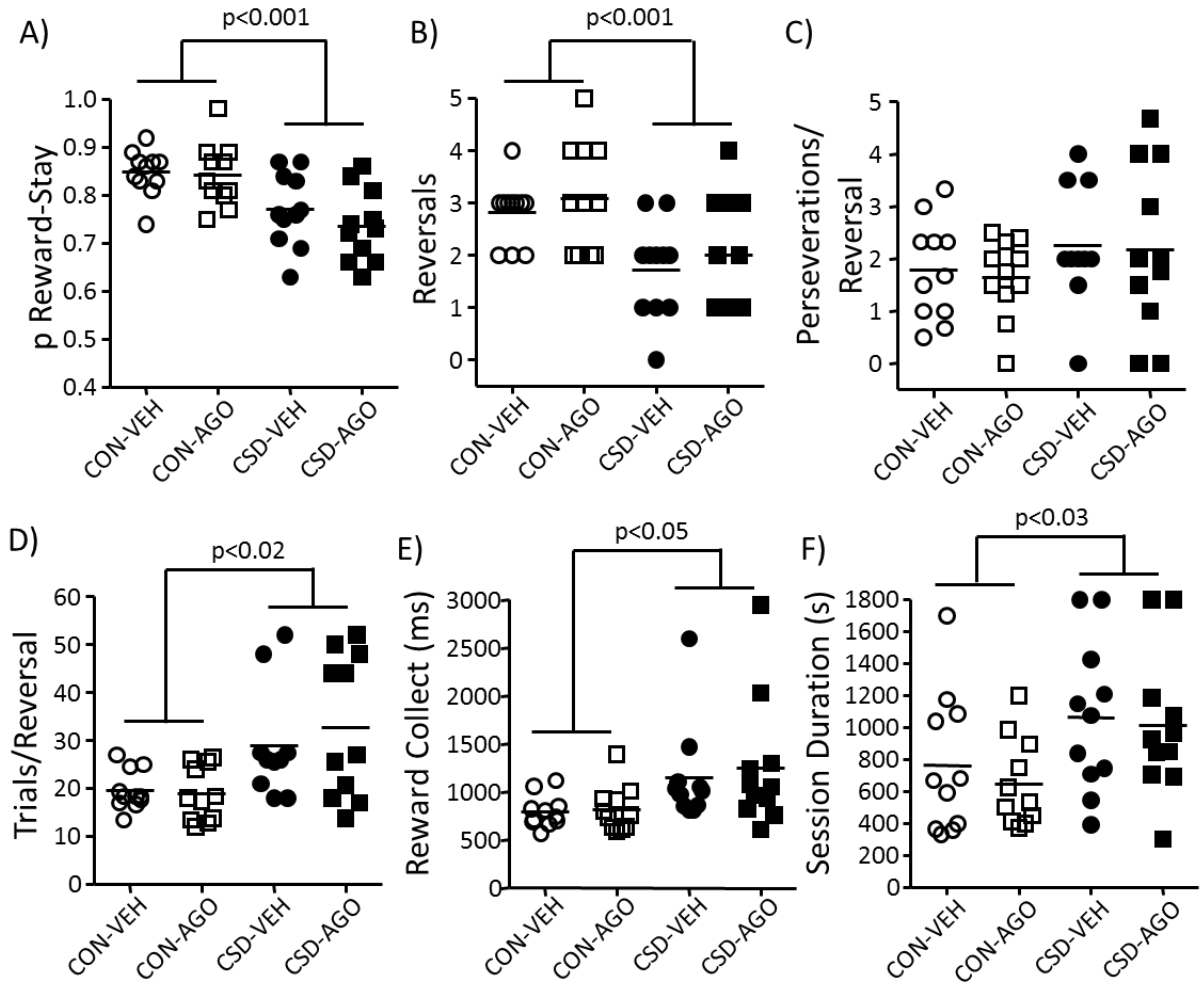

Figure 6. Study B: Effects of chronic social defeat and agomelatine (25 mg/kg per os) on mouse behaviour in the simple reversal learning test, at 4 days after completion of CSD and after 10 days of AGO. The test was conducted 1-2 hours after daily administration of AGO/VEH at the end of the inactive phase. Details of the experimental design are given in Figure 1. Eleven mice per group were tested. (A) Probability of reward-stay

responding. (B) Number of reversals completed. (C) Perseverations per reversal. (D) Trials per reversal. (E) Latency to collect reward from feeder. (F) Session duration. p values are for the main effect of Stress group in Stress group x Drug dose ANOVA.

At day 22, 7 days after the end of CSD and day 13 of AGO administration, mice were tested in the complex reversal test (Figure 7). There were main effects of Stress group, consistent with impaired test performance in CSD mice, for: number of reinforcements obtained (CON: 60±1, CSD: 52±11, p<0.002), probability of reward-stay responding (p<0.004, Fig. 7A), reversals completed (p<0.001, Fig. 7B), trials per reversal (p<0.03, Fig. 7D), average latency to collect reward (p<0.04) and session duration (p<0.0005, Fig. 7F). Negative feedback sensitivity was actually reduced in CSD mice relative to CON mice (p<0.02, Fig. 7E). In addition to the CSD effect on reversals completed, there was also a borderline non-significant effect of Drug (p<0.08); in Stress group-specific *t*-tests, CSD-AGO mice completed more reversals than did CSD-VEH mice (p<0.03; Fig. 7B). For perseverations per reversal, there was a Stress x Drug interaction (p<0.03, Fig. 7C), and post hoc tests identified that CSD-AGO mice perseverated more than each of the other three groups.

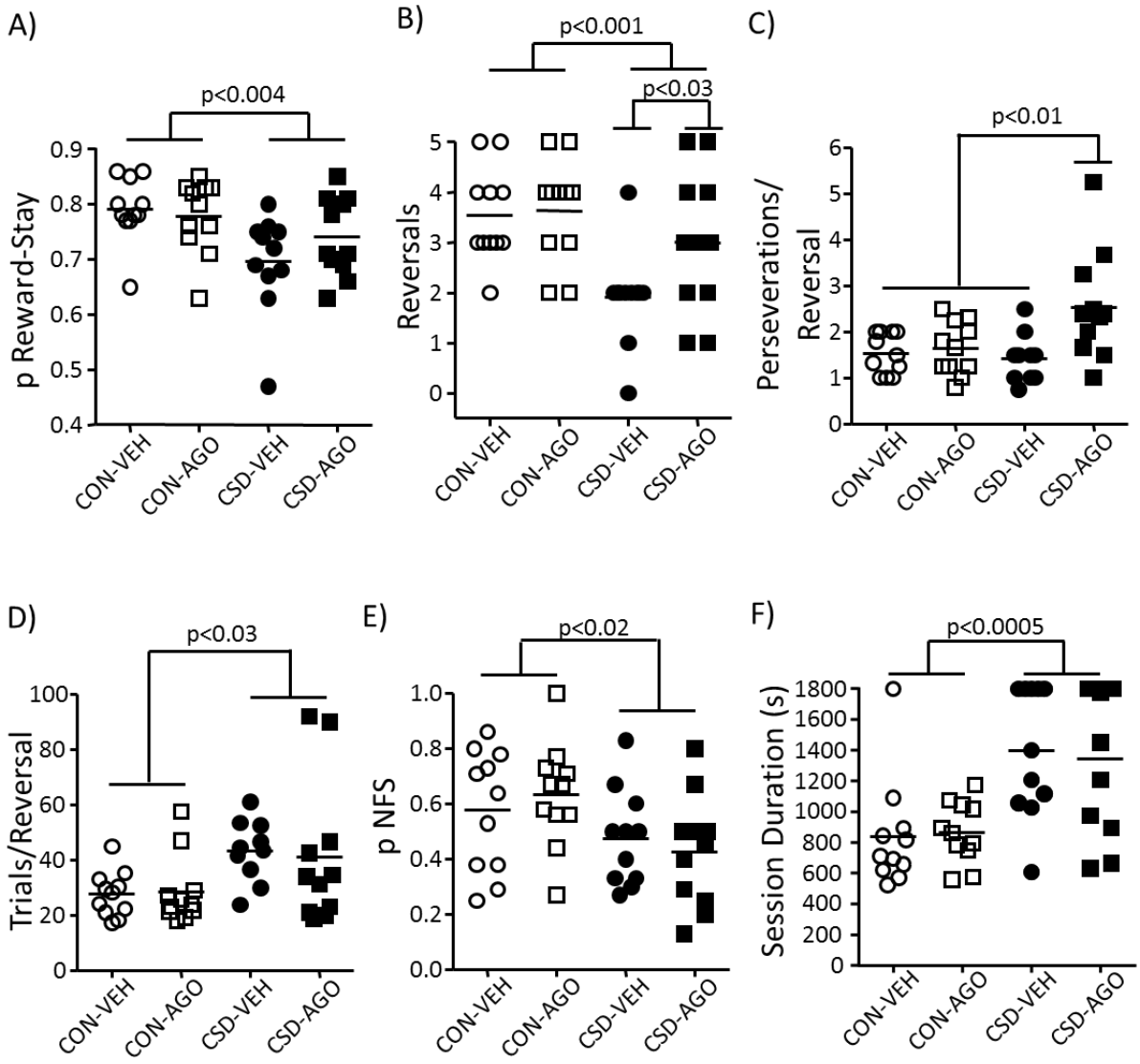

Figure 7. Study B: Effects of chronic social defeat and agomelatine (25 mg/kg per os) on mouse behaviour in the complex reversal learning test, at 7 days after completion of CSD and after 13 days of AGO. The test was conducted 1-2 hours after daily administration of AGO/VEH at the end of the inactive phase. Details of the

experimental design are given in Figure 1. Eleven mice per group were tested. (A) Probability of reward-stay responding. (B) Number of reversals completed. (C) Perseverations per reversal. (D) Trials per reversal. (E) Negative feedback sensitivity to non-reward of a correct response. (F) Session duration. In A, D-F, p values are for the main effect of Stress group in Stress group x Drug dose ANOVA. In B, there was a main effect of Stress group and borderline main effect of Drug group and Stress group-specific t-tests identified increased reversals due to AGO in CSD mice specifically. In C, there was a Stress group x Drug dose interaction, and p values are for post hoc Bonferroni pairwise comparisons.

Study C: CSD mice exhibit deficits in reward motivation and altered circadian rhythm, effects which do not respond to repeated agomelatine

In the first IntelliCage study (C1), saccharin- and water-directed visits, nosepokes and licks, were compared in CSD and CON mice (Figure 8 and Table S1). In the Stress x Day-block x Time-block ANOVA model, in cases of significant interactions of Stress x Day x Time and/or Stress x Day and/or Stress x Time, *a posteriori* Stress X Time ANOVAs were conducted for each day-block separately. Baseline (days -4 to 0) behaviour was similar in mice allocated at random to CON and CSD groups (Fig. 8A-D). As expected (e.g. (Cathomas et al., 2015a)), under baseline and CON conditions and primarily during the dark phase, mice had a moderate preference for the saccharin corner versus water corner in terms of visits and nosepokes, and a marked preference for saccharin over water in terms of licks. For saccharin visits (Table S1), CSD mice made less visits than CON mice during the dark phase at day-blocks CSD 1-5, 6-10 and 11-15. The same CSD effect pertained for water visits (Table S1). Together, these data suggest a general decrease in goal-directed activity. For saccharin nosepokes (Fig. 8A), CSD mice made less nosepokes than did CON mice during the dark phase at day-blocks CSD 1-5, 6-10 and 11-15. The same CSD effect pertained for water nosepokes (Fig. 8B), again consistent with a general decrease in appetitive behaviour. For saccharin licking (Fig. 8C), CSD mice made less licks than did CON mice during the dark phase at day-block CSD 11-15, consistent with a decrease in interest in saccharin by this stage. For water licks (Fig. 8D), CSD mice made more licks than CON mice during the light phase at day-blocks CSD 1-5 and 6-10, which possibly reflected increased activity and feeding during this period (see Discussion).

In the second IntelliCage study (C2), in which CSD-AGO and CSD-VEH mice were compared (Figure 9A-D and Table S2), the saccharin- and water-directed behavioural profiles of these mice were similar to those of the CSD mice in the first experiment. This was indicated by the main effect of Day-block in the Drug x Day-block x Time-block ANOVA model, attributable to a decrease in behaviour relative to baseline (days -4 to 0) in at least one CSD/post-CSD day-block, for dark-phase saccharin visits, dark-phase saccharin nosepokes (see Fig. 9A) and dark-phase saccharin licks (see Fig. 9C). Furthermore, light-phase water licks were increased in CSD/post-CSD day-blocks relative to baseline (see Fig. 9D). There were no significant effects involving Drug for any behavioural measure in these CSD mice.

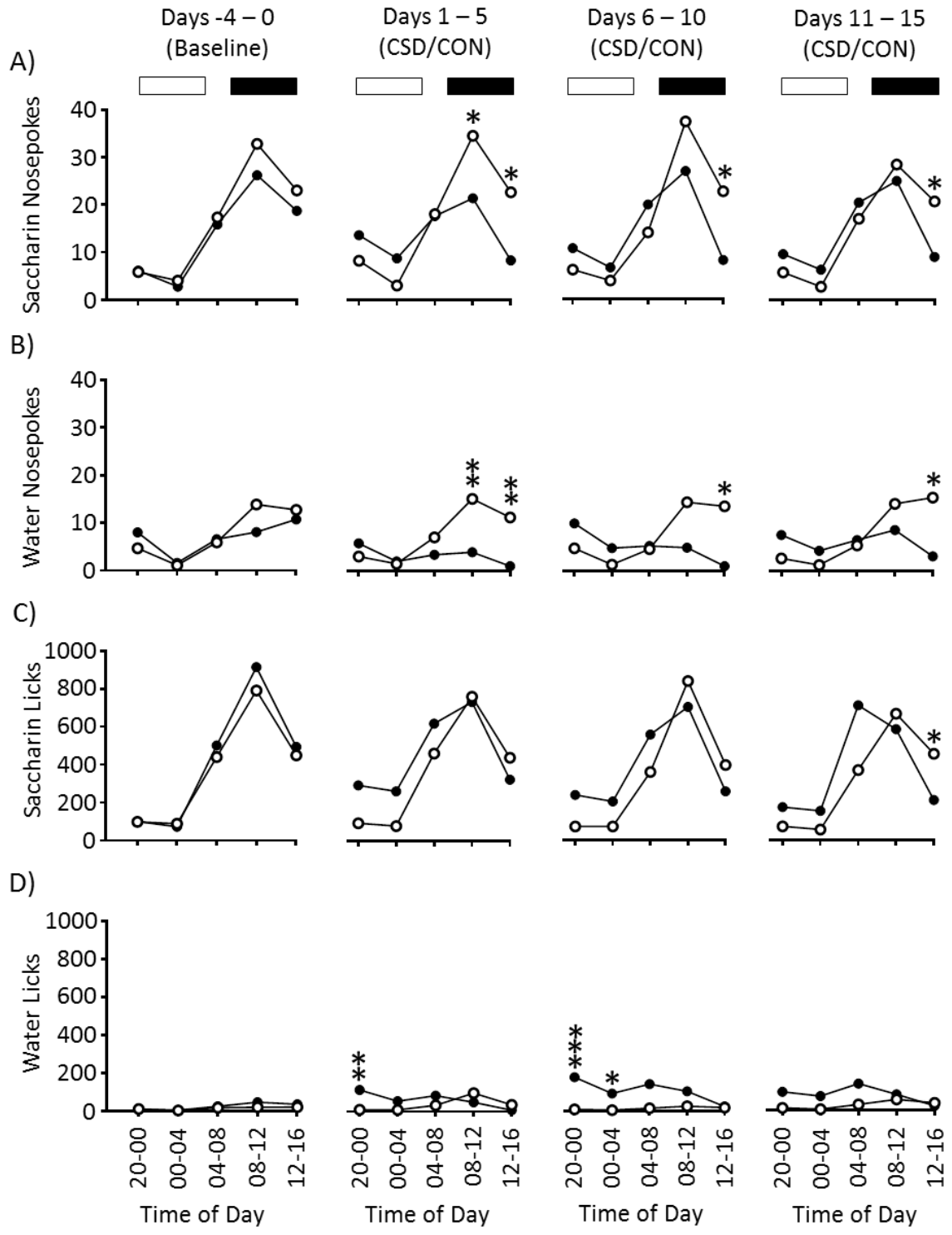

Figure 8. Study C: Effects of chronic social defeat on mouse behaviour relative to saccharin and water, as measured continuously in IntelliCage. A) Saccharin nosepokes. B) Water nosepokes. C) Saccharin licks. D) Water licks. Seven CSD and 11 CON mice were studied; one mouse from each group had to be excluded because of technical problems. Each data point is the overall mean of 5-day-mean values per mouse, obtained in 5 days prior to CSD/CON, CSD/CON days 1-5, 6-10 and 11-15, for each given 4-h period. Time periods were 20:00-00:00 and 00:00-04:00 during the light/inactive phase, 04:00-08:00 spanning the light-dark transition, and 08:00-12:00 and 12:00-16:00 during the dark/active phase. Daily CSD/CON procedures were carried out at 16:00-17:00 and the data obtained in the 3-4 h period thereafter were excluded from the analysis. Following Stress group x Time period interaction, * $p<0.05$, ** $p<0.01$, *** $p < 0.001$ are for CSD versus CON post hoc Bonferroni pairwise comparisons. For additional data see Table S1.

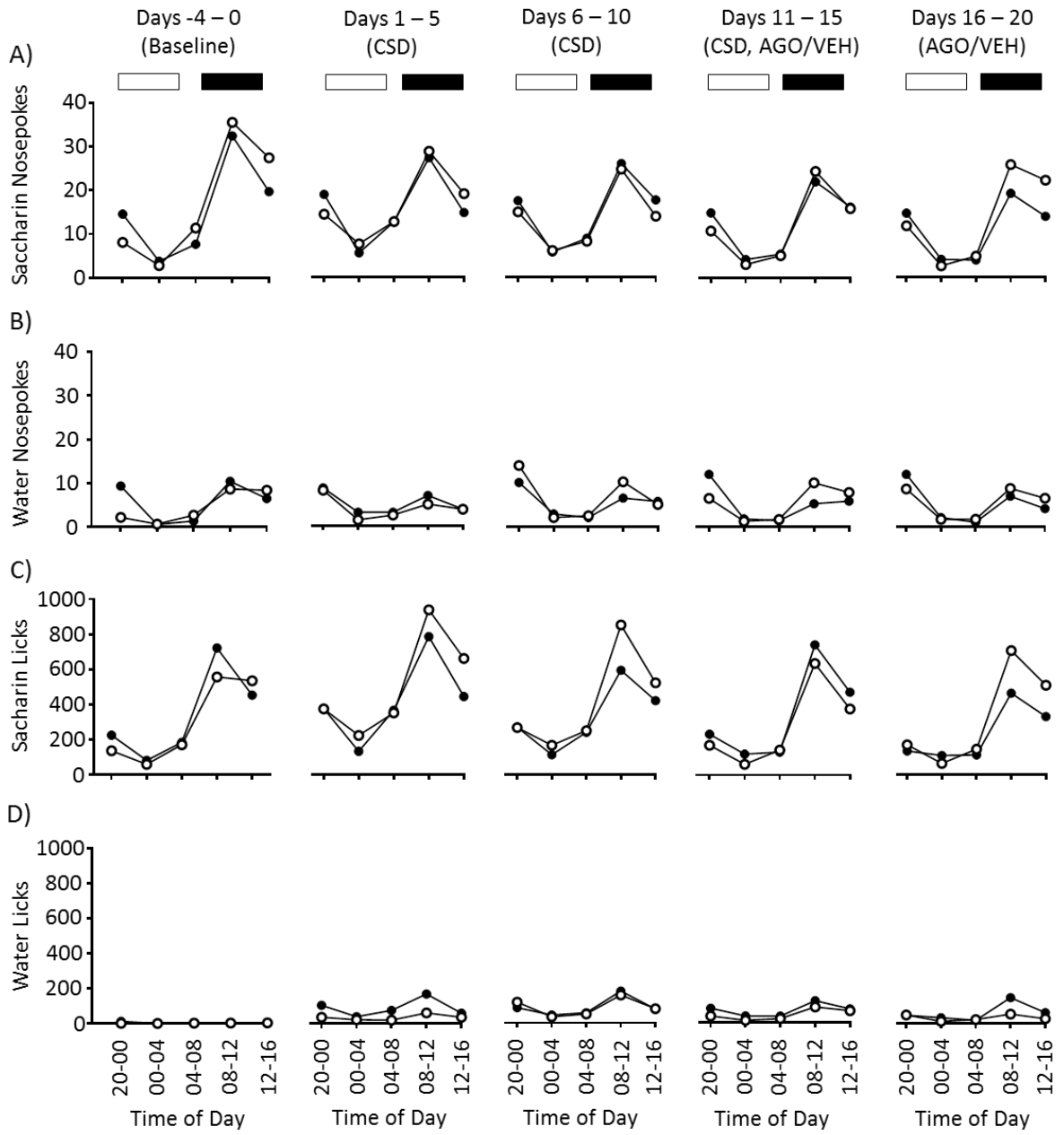

Figure 9. Study C: Effects of agomelatine (25 mg/kg per os) in mice exposed to chronic social defeat on behaviour relative to saccharin and water, as measured continuously in IntelliCage. A) Saccharin nosepokes. B) Water nosepokes. C) Saccharin licks. D) Water licks. Eight CSD and 8 CON mice were studied. Each data point is the overall mean of 5-day-mean values per mouse, obtained in 5 days prior to CSD, on CSD days 1-5 without AGO/VEH, on CSD days 6-10 with AGO/VEH administration starting on day 7, on CSD days 11-15 with AGO/VEH, and on post-CSD days 16-20 with AGO/VEH, for each given 4-h period. Time periods were 20:00-00:00 and 00:00-04:00 during the light/inactive phase, 04:00-08:00 spanning the light-dark transition, and 08:00-12:00 and 12:00-16:00 during the dark/active phase. Daily CSD was carried out at 16:00-17:00 and the data obtained in the 3-4 h period thereafter were excluded from the analysis, as were the data obtained at 04:00-08:00. AGO/VEH were administered at 06:00-07:00. For additional data see Table S2.

Discussion

In the present study we could demonstrate in discrete operant tests that exposure of mice to chronic psychosocial stress results in a decrease in motivation to exert physical effort for gustatory reward, and to decreased accuracy and negative feedback sensitivity when cognitive effort was required to obtain gustatory reward. In the home cage setting we could also demonstrate that appetitive, and to a lesser extent, consummatory responding to gustatory reward was decreased. The antidepressant agomelatine was effective in reducing the stress-induced deficit in complex reversals completed, and this co-occurred with an increased perseveration which was adaptive under the conditions of the test. These findings add considerably to the evidence that mice can provide useful models for the study of stress-induced disruption of the motivation and cognitive capacity to interact with stimuli that are normally rewarding, which is a core and common psychopathology in depression.

In the complex reversal learning (CRL) test, an acute dose of AGO at 25 mg/kg or 5-HT_{2C} antagonist, administered orally 1-2 hours prior to testing, led to an increase in the number of reversals completed by otherwise non-manipulated mice. Although neither measure responded significantly, each drug increased average reward-stay probability and decreased average negative feedback sensitivity (NFS), suggesting that these two additive effects underlay the observed consistent drug effect on reversals completed. In a previous study, a low, acute dose of the antidepressant escitalopram increased reward-stay, decreased NFS and increased reversals completed in mice in this CRL test (Ineichen et al., 2012). In a simple reversal learning test in rat, the 5-HT_{2C} antagonist SB 242084 improved reversal learning (Boulougouris et al., 2008). 5-HT_{2C} receptors are expressed in a number of forebrain regions important in the regulation of operant behaviour, including frontal cortex, amygdala, hippocampus, and dorsal and ventral striatum. 5-HT_{2C} antagonism, including AGO, disinhibits DA release into frontal cortex specifically (Di Giovanni et al., 2006; Millan, 2003). 5-HT_{2C} receptors are also expressed in monoamine cell-body regions, including ventral tegmental area (VTA) for dopamine (DA) and locus coeruleus for noradrenaline (NA) (Pompeiano et al., 1994; Racagni et al., 2011). In VTA, 5-HT_{2C} is expressed by GABA interneurons that project onto DA neurons (Eberle-Wang et al., 1997). AGO, acute and chronic, increases the number of spontaneously active VTA DA neurons and the bursting activity thereof (Chenu et al., 2013). Nonetheless, 5-HT2C antagonism does not disinhibit DA release in nucleus accumbens or dorsal striatum (Millan, 2003).

Chronic social defeat, as described previously (Kumar et al., 2013), resulted in increased food intake. This occurred in the absence of an effect on body weight, consistent with CSD inducing an increase in energy expenditure. In blood samples collected on day 16 from these same mice, there was a decrease in plasma leptin levels, as reported previously for CSD (Kumar et al., 2013). Leptin is an adipokine released from fat into the circulation. It is transported across the blood brain barrier where it acts to suppress feeding. It acts at homeostatic areas (e.g. brainstem, hypothalamus) as well as areas involved in reward-motivation such as VTA. The reduction in plasma leptin observed in CSD mice relative to CON mice under free-feeding conditions would be expected to contribute to their increased feeding by disinhibition. There was no consistent effect of CSD on the active form of ghrelin, which acts to promote feeding and has been reported to be increased by CSD and to underlie the increased feeding by CSD mice that, in contrast to the present study, exhibited increased body weight (Patterson et al., 2013). These findings confirmed the importance of providing CSD and CON mice with the group- and individual-specific feeding regimens that were required for maintaining the required level of baseline body weight, to establish appropriate conditions for operant testing.

Chronic social defeat was without effect on the principle measures of the progressive ratio schedule (PRS) test at CSD 8, and resulted in a decrease in responding by the final day of CSD, day 15.

In the same mice, tested in the simple reversal learning (SRL) test at day 19 and the CRL test at day 22, there were further effects of CSD on operant behaviour for reward. In the SRL test, CSD mice exhibited decreased reward-stay accuracy and, as a consequence of this, completed less reversals, requiring more trials per reversal. That these CSD effects were partly a consequence of reduced reward motivation – despite testing being conducted at 95% baseline body weight - was indicated by the increased latency to collect the sucrose-pellet after a correct response. In the CRL test, the decreases in reward-stay accuracy, reversals completed, trials required per reversal, and the increased latency to collect the reward, as observed in the SRL test, still pertained. Impaired ability to maintain accurate monitoring of the correct stimulus under cognitively effortful conditions might have contributed to these CSD effects, as might reduced reward expectancy. The effects co-occurred with a decrease in negative feedback sensitivity (NFS). Non-manipulated mice, in contrast to humans, exhibit high NFS, which suggests that mice are indeed able to monitor reward expectancy in the CRL test, experience it as aversive when an expected reinforcement is withheld and, in contrast to healthy humans, cannot inhibit the disposition to switch stimuli on the next trial (Ineichen et al., 2012). Against this background, the reduced NFS in CSD mice could indicate reduced cognitive ability to monitor reward expectancy, reduced negative emotionality when an expected reinforcement is withheld, or reduced reward expectancy. In the human PRL test, depressed patients exhibit intact reward-stay accuracy and reversals completed and increased NFS, relative to healthy controls (Taylor Tavares et al., 2008). Therefore, the constellation of CSD effects in the mouse CRL test is the opposite of the constellation of effects of depression in the human PRL test. Nonetheless, the mouse CRL test provides clear evidence for impaired reward-directed behaviour under cognitively demanding conditions in mice that had been exposed to CSD, which is certainly relevant to the major depression psychopathology of reduced interest in daily activities.

Evidence from an ecological setting (Cathomas et al., 2015b) that CSD reduces motivation to engage in appetitive behaviour, complementary to that obtained in the discrete testing conditions pertaining in the PRS test, was provided by the IntelliCage experiment. CSD mice developed reduced goal-directed behaviour (visits, nosepokes) for saccharin and water during the active period of their circadian cycle from the first days of the 15-day CSD procedure. Towards the end of CSD they had also developed reduced consumption (licks) of saccharin during the active period. During the inactive period, the water consumption of CSD mice increased, possibly indicating that they were more active than CON mice during this period and eating more food, which stimulates drinking.

Agomelatine, using the dose at which acute administration led to increased reversals completed in the CRL test in non-manipulated mice, also exerted some deficit-reducing effects when administered repeatedly in CSD mice. Firstly, there was a tendency to increase motivation for reward in the PRS test conducted after 6 days of repeated AGO, in CSD mice. Deficient motivation in the PRS test is also induced by NAcc DA depletion (Bergamini et al., Submitted manuscript), suggesting AGO effects on VTA DA neurons (Chenu et al., 2013) could have contributed to the partial AGO effect on motivation in CSD mice. Whilst there were no effects of 10 days of AGO in the SRL test, 13 days of AGO resulted in an increase in reversals completed in the CRL test in CSD mice specifically. Furthermore, this co-occurred with an increase in the average number of perseverations per reversal in CSD-AGO mice compared with each of the other groups. In the CRL test, where some correct responses are not rewarded and could therefore be regarded - incorrectly - as a reversal, it is actually adaptive to be perseverative and thereby to increase the ability to differentiate non-rewarded correct responses from actual reversals. Two perseverations per reversal are sufficient: CSD-AGO mice made 2-3 on average and each of the other groups made 1-2. This co-occurred with CSD-AGO mice also exhibiting the lowest average NFS score of each of the groups. Therefore, repeated AGO

produced increased perseveration specifically in CSD mice and specifically in the CRL test. This might represent a first indicator of restoration of normal reward expectancy and motivation in CSD mice, due to repeated administration of AGO. With regards to underlying mechanisms, as described above, the medial prefrontal cortex is a major region in the regulation of perseveration, and both 5-HT_{2C} antagonism and increased DA receptor-specific agonism in this region could mediate the positive effects of AGO observed (Boulougouris et al., 2008; Floresco et al., 2006; Millan, 2003). There were no effects of AGO on the CSD-induced home-cage deficits in appetitive behaviour and shifts in circadian activity, perhaps indicating that its effects are specific to physically effortful (cf. PRS test) or cognitively effortful (cf. CRL test). Nonetheless, this study has provided original and substantive evidence that chronic psychosocial stress can be combined with operant tests of reward-directed behaviour to provide mouse models for the deficit in reward motivation that is a major psychopathology of depression.

Supplementary information

Table S1. Summary of IntelliCage behaviour in Experiment C1, according to reward, stress group, day-block and time-period

	Light Phase (20-00 h, 00-04 h)	Dark Phase (08-12 h, 12-16 h)
Reward/Measure/Day-block	**CON/CSD**	**CON/CSD**
Saccharin Visits -4 to 0	2.22±0.94/1.80±1.00	11.60±3.88/9.20±2.43
Saccharin Visits 1-5	1.91±0.87/4.97±1.13	11.42±6.70/5.14±2.81*
Saccharin Visits 6-10	2.20±1.68/3.83±1.68	10.58±5.45/5.40±2.85*
Saccharin Visits 11-15	1.75±0.81/4.07±2.39	10.29±3.41/4.99±3.27**
Saccharin Nosepokes -4 to 0	4.00±2.55/2.74±1.66	23±11.90/18.71±6.73
Saccharin Nosepokes 1-5	3.02±1.28/8.74±3.07	22.71±12.61/8.31±4.89*
Saccharin Nosepokes 6-10	3.95±4.40/6.74±2.34	23.04±17.34/8.34±5.81*
Saccharin Nosepokes 11-15	2.76±1.39/6.36±3.36	20.80±10.07/9.04±6.61*
Saccharin Licks -4 to 0	89.16±55.43/72.69±75.55	449.55±239.39/493.86±136.51
Saccharin Licks 1-5	77.60±63.48/261.03±141.84	438.87±241.30/322.91±259.64
Saccharin Licks 6-10	73.02±53.62/206.26±85.20	401.15±226.82/260.66±254.38
Saccharin Licks 11-15	54.65±37.93/153.14±118.60	454.47±193.53/210.41±209.70*
Water Visits -4 to 0	0.84±0.44/0.97±0.65	6.27±4.22/4.75±2.62
Water Visits 1-5	1.07±0.75/1.86±1.16	7.33±4.95/1.03±0.61*
Water Visits 6-10	1.65±1.19/6.57±3.46**	6.39±5.05/1.69±1.29**
Water Visits 11-15	1.13±1.06/4.75±3.70*	7.83±4.11/2.39±2.05**
Water Nosepokes -4 to 0	1.13±1.22/1.60±1.60	12.75±18.38/10.74±10.88
Water Nosepokes 1-5	1.51±1.28/2.03±1.08	11.27±11.77/1.03±1.56**
Water Nosepokes 6-10	1.38±1.16/4.89±3.55	13.78±18.66/1.06±0.98*
Water Nosepokes 11-15	1.24±1.06/4.25±2.94	15.45±16.40/3.09±2.71*
Water Licks -4 to 0	2.84±3.94/3.43±6.31	20.60±30.60/35.34±67.25
Water Licks 1-5	10.20±14.14/114.23±120.18**	36.15±63.86/8.23±9.38
Water Licks 6-10	8.04±11.53/178.74±193.32***	16.85±32.51/23.09±44.37
Water Licks 11-15	5.46±7.39/74.18±107.45	38.95±70.58/22.77±30.57
Preference Visits -4 to 0	73.39±6.47/65.20±17.70	66.81±9.04/67.94±11.96
Preference Visits 1-5	68.96±14.08/74.09±11.20	62.71±9.25/81.66±9.78**
Preference Visits 6-10	69.27±9.89/58.84±8.63*	67.13±8.94/79.64±11.39*
Preference Visits 11-15	70.95±17.40/59.42±16.45	59.42±10.86/70.58±17.10
Preference Nosepokes -4 to 0	80.61±9.61/67.03±22.96	72.14±16.14/70.02±15.43
Preference Nosepokes 1-5	71.23±14.89/81.60±9.55	71.38±10.34/88.10±11.34*
Preference Nosepokes 6-10	71.52±18.67/62.51±13.15	71.26±16.39/87.88±10.21
Preference Nosepokes 11-15	73.86±22.14/58.96±23.84	66.33±16.60/74.89±17.83
Preference Licks -4 to 0	97.60±3.31/91.46±18.28	96.24±3.67/93.40±12.56
Preference Licks 1-5	92.89±8.48/75.55±17.50*	93.88±8.80/93.94±7.40
Preference Licks 6-10	91.76±14.15/77.46±15.67	96.22±8.03/96.01±6.17
Preference Licks 11-15	87.53±23.70/70.17±27.79	92.69±11.53/87.10±16.35

Table S2. Summary of IntelliCage behaviour in Experiment C2, according to reward, drug group, day-block and time-period

Reward/Measure/Day-block	Light Phase (20-00 h, 00-04 h) VEH/AGO	Dark Phase (08-12 h, 12-16 h) VEH/AGO
Saccharin Visits -4 to 0	1.85±1.11/2.45±1.23	14.30±6.32/12.28±3.93
Saccharin Visits 1-5	4.23±1.48/3.45±1.47	11.03±3.53/9.43±2.71
Saccharin Visits 6-10	2.88±1.23/3.10±1.89	8.23±4.66/12.45±6.35
Saccharin Visits 11-15	1.58±0.94/2.50±0.87	10.93±4.30/10.78±3.08
Saccharin Visits 16-20	1.63±0.74/2.35±1.44	11.28±3.12/8.69±2.15
Saccharin Nosepokes -4 to 0	2.83±1.59/3.78±2.12	27.45±21.47/19.73±6.62
Saccharin Nosepokes 1-5	7.65±3.60/5.60±2.35	19.15±11.10/14.80±4.32
Saccharin Nosepokes 6-10	6.25±3.67/5.88±3.93	14.18±7.59/17.95±5.45
Saccharin Nosepokes 11-15	3.05±1.65/4.18±1.41	15.88±9.59/16.25±5.43
Saccharin Nosepokes 16-20	2.67±1.60/4.16±2.78	11.43±13.7114.08±4.87
Saccharin Licks -4 to 0	60.70±58.53/82.95±46.83	557.63±149.45/723.08±249.23
Saccharin Licks 1-5	225.93±163.18/135.23±34.58	666.23±280.45/448.53±237.54
Saccharin Licks 6-10	170.58±120.48/115.08±67.63	532.45±316.55/428.93±198.57
Saccharin Licks 11-15	62.43±44.38/120.13±74.29	379.25±178.36/475.83±200.32
Saccharin Licks 16-20	64.43±50.11/110.17±61.19	515.45±220.31/334.64±120.43
Water Visits -4 to 0	0.68±0.46/0.78±0.37	6.40±3.53/4.98±2.70
Water Visits 1-5	1.08±0.59/1.58±1.27	3.48±1.76/3.83±2.01
Water Visits 6-10	1.40±0.57/1.78±1.12	3.95±2.26/5.70±3.94
Water Visits 11-15	0.85±0.48/1.05±0.62	6.50±3.99/5.35±3.59
Water Visits 16-20	1.38±0.43/1.35±0.61	5.48±2.14/3.30±2.33
Water Nosepokes -4 to 0	0.70±0.54/0.63±0.27	8.43±7.02/6.53±4.55
Water Nosepokes 1-5	1.48±1.34/3.20±3.11	3.93±3.87/3.93±3.14
Water Nosepokes 6-10	2.10±1.36/2.9±2.45	5.13±4.88/5.80±5.45
Water Nosepokes 11-15	1.33±1.26/1.85±1.22	7.93±7.84/5.90±3.77
Water Nosepokes 16-20	1.68±0.92/2.08±0.88	6.55±3.26/4.16±3.3.7
Water Licks -4 to 0	0.08±0.20/0.80±1.44	3.45±5.37/4.68±10.06
Water Licks 1-5	22.13±31.11/39.45±59.39	36.05±53.63/59.43±79.29
Water Licks 6-10	35.73±35.02/45.16±86.44	81.78±131.70/80.75±176.67
Water Licks 11-15	12.55±15.52/37.63±52.55	67.70±96.33/78.65±130.54
Water Licks 16-20	12.53±14.98/33.61±42.62	27.68±50.65/62.73±111.84
Preference Visits -4 to 0	73.22±15.32/78.09±9.62	69.16±10.86/73.20±10.11
Preference Visits 1-5	80.37±8.49/71.44±16.03	76.14±9.15/71.17±13.85
Preference Visits 6-10	66.69±8.13/64.97±13.66	66.75±14.22/69.34±17.49
Preference Visits 11-15	67.29±14.83/72.18±10.75	64.06±9.19/69.65±11.39
Preference Visits 16-20	52.40±14.96/65.34±20.03	67.15±9.47/75.02±12.52
Preference Nosepokes -4 to 0	79.91±19.27/85.45±8.90	75.11±15.51/75.91±13.37
Preference Nosepokes 1-5	85.25±8.29/69.91±23.54	84.81±8.62/80.32±14.00
Preference Nosepokes 6-10	72.38±12.86/70.05±19.69	73.89±15.81/77.09±20.41
Preference Nosepokes 11-15	75.63±15.30/71.88±13.19	68.59±14.20/75.27±11.85
Preference Nosepokes 16-20	59.58±19.56/67.70±17.59	74.28±13.22/80.07±13.98

Preference Licks -4 to 0	99.46±1.42/98.59±2.90	99.32±1.23/99.04±2.01
Preference Licks 1-5	91.74±10.22/85.41±18.89	94.42±6.73/89.31±17.10
Preference Licks 6-10	86.29±11.62/83.69±27.40	83.45±20.18/87.94±25.78
Preference Licks 11-15	85.95±13.64/84.04±19.60	85.57±15.87/88.72±15.83
Preference Licks 16-20	88.28±9.08/81.37±22.13	93.84±10.05/89.29±19.07

General discussion

1. Stress, inflammation and dopamine: a route to depression

In the present PhD thesis the effects of neurochemical, environmental and pharmacological manipulations on reward and punishment processing in mice have been investigated.

In Study A it was demonstrated that chronic DA depletion in the NAcc, achieved using the catecholaminergic neurotoxin 6-OHDA, impacts on mouse motivation, namely impairing approach behaviour towards positive stimuli (i.e. palatable rewards) and reducing escape/avoid behaviour towards negative events (i.e. footshocks). Specifically, NAcc DA depletion led to decreased responding for rewards in the progressive ratio schedule (PRS) test, and to a stimulus with changing association with gustatory reward in the learned non-reward (LNR) test. It reduced responding to footshock recently experienced as uncontrollable in the learned helplessness (LH) test, and to footshock that could only be avoided-escaped by physical effort in the treadmill test. In contrast to these motivational effects on operant behaviour, NAcc DA depletion did not affect the learning of or memory for stimulus-stimulus learning in the Pavlovian fear conditioning test. In Study B I have demonstrated that CSD leads to reduced DA turnover and *c-fos* expression in the NAcc, and to reduced sensitivity to pharmacologically-induced increased synaptic DA availability, in terms of locomotion/exploration and NAcc immediate-early gene expression. Furthermore, in Studies B and C I have demonstrated that CSD leads to reduced reward-directed behaviour in the LNR and PRS tests and also in the probabilistic reversal learning (PRL) test. Therefore, integrating the findings from these two studies provides robust evidence that CSD effects on NAcc DA neurotransmission underlies the CSD-induced deficits in reward-directed behaviour, particularly in terms of impaired motivation. With regard to CSD-induced effects on behaviour towards negative events, a previous study in our laboratory has demonstrated that CSD leads to increased fear conditioning, to decreased 2-way avoid-escape responding (i.e. increased helplessness) similar to that induced by NAcc DA depletion, and to reduced running in the treadmill, again similar to that induced by NAcc DA depletion (Azzinnari et al., 2014). Extrapolating from Study A, it would appear that CSD induces motivational deficits with respect to positive- and negative-valence stimuli and that altered NAcc DA function contributes significantly to these deficits. Furthermore, given that CSD increases fear conditioning whereas NAcc DA depletion is without effect, CSD effects clearly extend beyond the VTA-NAcc DA pathway with altered amygdala function being a major candidate (Azzinnari et al., 2014).

Thus, the current PhD thesis, adopting an RDoC-informed approach on translational research for depression, suggests that behavioural domains pertaining to positive and negative valence system (PVS and NVS) are directly affected by social stress and inhibition of DA transmission. Moreover, as presented in Study B, stress-induced inflammation represents a candidate mechanism linking stress, mesolimbic DA dysregulation and the observed behavioural changes.

1.1 Cell-mediated immune activation and inflammation in depression

As mentioned in section 3.1 of Introduction, depression is characterized by inflammation and cell-mediated immune activation (Maes, 2011). Indeed, it has been shown that depression is characterized by higher numbers of peripheral blood mononuclear cells (PBMCs), including both lymphocytes and monocytes (Maes et al., 1992; Seidel et al., 1996). Moreover, Chen et al. (2011) showed an increase in peripheral Th17 cell number, a higher level of mRNA expression for retinoic acid-related orphan receptor-γt (RORγt), the specific transcription factor of Th17 cells, in peripheral blood lymphocytes, and increased serum concentration of IL-17, in depressed subjects. In my thesis I demonstrate that CSD leads to a higher number of splenic myeloid cells (namely, granulocytes and

inflammatory monocytes) and Th17 cells. It has been shown proposed that stress increases the production of myeloid cells in the bone marrow, that then traffic to the blood, spleen and brain (Reader et al., 2015; Wohleb et al., 2014b), thus contributing to the increased inflammatory cytokines levels observed in tissues and blood. Interestingly, chronic stress-induced leucocytosis is associated with non-response to the anti-inflammatory effects of glucocorticoid (GC) and a transcriptional profile that is consistent with the expansion and priming of myeloid-derived cells, in both humans and rodents (Cohen et al., 2012; Powell et al., 2013; Reader et al., 2015). In addition to immune system activation, acute stress is also known to induce suppression of the immune system, in part by the anti-inflammatory effects of increased GC. Stressful life experiences, including long-term threat, are associated with a higher probability of developing a cold following exposure to a rhinovirus (Cohen et al., 2012; Miller et al., 2008b). This effect has been proposed to depend on chronic stress-induced GC resistance (Engler et al., 2004), resulting in reduced control over the inflammatory response to the viral infection, thus leading to a greater expression of the disease (Cohen et al., 2012). Interestingly, in Study B, the reduced MHC II surface expression on splenic myeloid cells in CSD mice is suggestive of stress-induced immunosuppression. Together with leucocytosis, depressed subjects also show increased levels of inflammation and immune response biomarkers in the blood, including C-reactive protein (CRP) and the pro-inflammatory cytokines TNF-α and IL-6 (Lopresti et al., 2014). Interestingly, studies from this laboratory demonstrate that CSD leads to increased peripheral levels of TNF-α, IL-6, IFNγ (Azzinnari et al., 2014; Fuertig et al., in prep). These effects co-occur with CSD-induced marked adrenomegaly, suggesting chronic activation of the hypothalamic-pituitary-adrenal axis (Azzinnari et al., 2014). I have shown that myeloid cells in the spleen express higher levels of TNF-α and IFNγ (Study B). Oxidative stress markers (e.g. superoxide dismutase, 8-Hydroxy-2-deoxyguanosine (8-OHdG)) have also been shown to be increased in the blood of depressed patients (Lopresti et al., 2014). To assess oxidative stress response to CSD, I measured plasma levels of inducible nitric oxide synthase (iNOS), and found this to be increased in CSD mice (Study B).

Inflammatory conditions are known to regulate the metabolism of tryptophan (Schwarcz et al., 2012). Tryptophan is converted into kynurenine (KYN) by the enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). KYN is further metabolized into other tryptophan catabolites (TRYCATs), namely 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HANA), through the enzymatic activity of kynurenine 3-monooxygenase (KMO) and kynureninase (Kynu). KYN can also be converted into kynurenic acid (KYNA) by kynurenine aminotransferases (KATs). The neuroactive metabolite quinolinic acid (QA) is also formed from 3-HK with the enzymatic participation of 3-hydroxyanthranilate oxygenase (3-HAO) (Colin-Gonzalez et al., 2013). KYN and 3-HK can be transported across the blood-brain barrier (BBB); indeed about 60% of brain KYN is derived from the periphery. Conversely, KYNA and QA cannot be transported across the BBB (Schwarcz et al., 2012) and need to be formed in the brain. In the brain, tryptophan degradation takes place mainly in microglia and astrocytes (Myint and Kim, 2014), with microglia cells producing KYN, 3-HK and QA and astrocytes being a major source of KYNA (Schwarcz et al., 2012). In addition, astrocytes can metabolize QUIN produced by microglia (Guillemin et al., 2001), thus controlling brain levels of QA. During inflammatory conditions, pro-inflammatory cytokines and reactive oxygen species (ROS) can enhance the activity of the enzyme IDO in extrahepatic tissues (lungs, placenta, kidneys, spleen, blood and brain) and of TDO in the liver and brain (Lestage et al., 2002; Walker et al., 2013). Although IDO expression is directly regulated by inflammatory molecules (e.g. IFN-γ), TDO up-regulation is mainly mediated by increased GC secretion (Campbell et al., 2014). Interestingly, the KYN pathway of tryptophan degradation is activated directly by inflammatory factors (Gibney et al., 2013) as well as

by environmental stress (Agudelo et al., 2014; Liu et al., 2013). This laboratory has recently demonstrated that CSD increases plasma and brain levels of KYN and 3-HK (Fuertig et al., in prep). In Study B of my thesis I demonstrate that the increased levels of TRYCATs might stem from the activation of KYN pathway enzymes in the liver, primarily under the rate-limiting factor of increased TDO. Interestingly, there are reports of KYN pathway activation in depression, both in the periphery and in the brain (Steiner et al., 2011; Sublette et al., 2011). Moreover, effective antidepressant treatments have been shown to modulate the KYN pathway, shifting KYN TRYCATs balance towards metabolites with a neuroprotective profile (Guloksuz et al., 2015).

Thus, CSD-induced peripheral immune response can activate the KYN pathway in the liver, and this, possibly in combination with increased KYN pathway activity in other tissues, leads to increased levels of KYN and 3-HK in the blood and translocation to the brain, where they can be converted to QA by microglia cells, with the latter increased in specific brain regions including the VTA (see Study B). In the brain, TRYCATs can exert a number of effects on neural and glial physiology, thus influencing the occurrence of behavioural changes.

1.2 Impact of Kynurenine pathway activation on brain cells

TRYCATs exert a plethora of effects that could contribute to the pathophysiology of depression, including modulation of neuronal cell death, glutamate transmission, and neuroinflammation (Myint and Kim, 2014). The KYN metabolites contribute directly to the neuroprotective/neurodegenerative changes in the brain through the interaction with several neurotransmitter systems and cell types.

KYNA and QA exert opposite effects on brain physiology. KYNA acts as an antagonist on glutamate NMDA receptors and on α7 nicotinic acetylcholine receptors (α7nAChR) (Schwarcz et al., 2012). In addition, KYNA can be considered as a potential endogenous antioxidant, through its free-radical scavenging activity (Lugo-Huitron et al., 2011). Conversely, QA is an agonist on NMDAR and is a source of oxidative stress (Rios and Santamaria, 1991). Since QA is a strong NMDAR agonist, sustained activation of NMDAR by QA may induce neuronal excitotoxicity through increased intracellular calcium concentration (Vandresen-Filho et al., 2015). It has been shown that when a single dose of QA is administered to the striatum, a reduction in the number of striatal neurons, with a decrement in the GABAergic neuronal population, is observed (Moresco et al., 2008; Perez-De La Cruz et al., 2012).

Regarding the effect of 3-HK on brain cells, there is currently no consensus with respect to whether it is primarily neuroprotective or neurodegenerative (Colin-Gonzalez et al., 2014). Under homeostatic conditions, 3-HK acts as a modulatory metabolite, with a major function of maintaining cellular redox homeostasis (Colin-Gonzalez et al., 2013). *In vitro* studies suggest that high levels of 3-HK can induce apoptotic cell death in cultures of striatal and cortical neurons, and in cerebellar granule cells (Chiarugi et al., 2001; Colin-Gonzalez et al., 2013). However, it is debated as to whether such effects are of relevance to *in vivo* effects of high 3-HK levels in brain (Colin-Gonzalez et al., 2014). Nakagami et al. (1996) showed that intra-striatal injection of 3-HK induces tissue damage around the injected site, but the cellular mechanism mediating its effects are unclear. It has been shown that the striatum of animals infused with 3-HK exhibits moderate levels of lipid and protein oxidation at a few hours post-lesion and that these effects are diminished across longer time intervals (days), such that long-term behavioural alterations or morphological changes in the injected striata are absent (Colin-Gonzalez et al., 2014). These data suggest that whilst 3-HK might exert pro-oxidant actions under certain conditions, it acts more as a redox modulatory molecule than as a neurotoxic metabolite (Colin-Gonzalez et al., 2014).

Thus, although CSD results in activation of the KYN pathway in the periphery, and blood and brain levels of KYN and 3-HK are increased in CSD mice (Fuertig et al., in prep), investigating the effects of CSD on brain levels of QA will be essential to understanding of the extent to which KYN-pathway activation is a neuropathological process affecting brain cell physiology (e.g. neurons, oligodendrocytes, astrocytes), and inducing behavioural changes. CSD-induced increase in QA levels might affect the physiology of several neuronal populations, including DAergic cells and inhibitory GABAergic interneurons. Interestingly, QA influences several neurotransmitter systems in the striatum, including DA and serotonin levels (Jamwal et al., 2015). Moreover, Feng et al. (2014) showed that intra-striatal injection of QA induces motor and cognitive impairments that correlate with a reduced number of striatal (DARPP-32^{+}) projection neurons and parvalbumin (Pvalb)$^{+}$ GABAergic interneurons. However, the degenerative effects of QA are not only restricted to neurons and also include astrocytes (Guillemin et al., 2005) and oligodendrocytes (Cammer, 2001; Sundaram et al., 2014). Using whole-transcriptome RNA sequencing (RNA-seq), this laboratory has demonstrated that CSD mice show lower expression levels of GABA interneuron markers (e.g. Pvalb) and of oligodendrocyte-myelin genes (e.g. *Mag*, *Mobp*, *Opalin*, *Aspa*), in the amygdala (Azzinnari et al., 2014). Thus, it can by hypothesized that CSD-induced activation of the KYN pathway includes increased brain levels of QA, which in turn could affect the function/survival of DAergic and GABAergic neurons, and of oligodendrocytes.

As indicated above, QA is an agonist at NMDAR, and its detrimental effects on brain cells might be mediated by excitotoxic mechanisms (Schwarcz et al., 2012). It has been shown that QA stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes (Tavares et al., 2002). These effects could contribute to increase extracellular glutamate concentrations, which then lead to overstimulation of the glutamatergic system (Tavares et al., 2002). Interestingly, depressive-like behaviours induced by the inflammatory toxin lipopolysaccharide (LPS) can be prevented by co-administration of the NMDAR antagonist and experimental anti-depressant, ketamine (Walker et al., 2013). This suggests that LPS-induces an increase in QA that then induces depressive-like behaviours, such that pharmacological blockade of NMDAR can prevent their onset. These findings are highly relevant for depression research, given the now fairly numerous reports that ketamine is efficacious as a rapid-onset therapy for treatment-resistant depression (Kirby, 2015; Yang et al., 2015; Zarate et al., 2006). This laboratory has shown that inhibition of the KYN pathway using an IDO1 inhibitor reverses CSD-induced behavioural changes (Fuertig et al., in prep), suggesting that activation of the KYN pathway is not only a marker for immune system activation but plays a causal role in CSD-induced behavioural changes.

Therefore, a link can be made between these and previous findings with the CSD mouse model and the hypothesis that stress-induced activation of the KYN pathway, and the consequent increase in brain QA levels, contributes to the aetiopathophysiology of depression-relevant behaviours. Accordingly, targeting of this pathway represents a promising approach for the identification of effective antidepressant pharmacological treatments.

2. Limitations of the thesis and outlook

This thesis provides evidence for the psycho-neuro-immune hypothesis for depression, suggesting that psychosocial stress activates the immune system, which in turn affects dopamine transmission and disrupts reward processing. In Study A, a causal relationship between reduced DA signaling in the NAcc and disrupted processing of positive and aversive stimuli has been demonstrated, providing indirect evidence that some of the same behavioural deficits observed in CSD mice might be underlain by the DA deregulation also observed in CSD mice. Moreover, this laboratory has

conducted a study demonstrating that an inhibitor of IDO1 reverses the increases in KYN and 3-HK plasma and brain levels and conditioned fear expression in CSD mice (Fuertig et al., in prep). This constitutes further support for the existence of a causal link between CSD-induced inflammation and behavioural changes. However, despite the aforementioned findings, direct evidence for a causal relationship linking CSD-induced inflammation to DA signaling deregulation is still lacking. Further investigations will need to address this issue. For example, this should include experiments assessing the effects of KYN pathway blockade on markers of DA signalling that are affected by CSD (i.e. NAcc DA turnover and behavioural sensitivity to GBR 12909).

Another critical question regards the potential contribution of the different TRYCATs to the observed brain and behavioural effects. Studies from this laboratory have demonstrated that KYN and 3-HK brain levels are increased in CSD mice, but CSD effects on QA brain levels could not be determined to-date due to the insufficient sensitivity for QA of the LC-MS/MS methodology used (Fuertig et al., in prep). To overcome this issue, we have recently established an immunohistochemical method for detection of QA in brain cells, based on a human report (Steiner et al., 2011). Thus, using immunohistochemistry it will be possible to assess CSD effects on brain QA levels. In the case that both 3-HK and QA brain levels are increased by CSD, brain region-specific infusion of both compounds followed by behavioural testing would provide important indications regarding their contributions to the CSD model. Given the findings of this thesis, then the VTA-NAcc pathway would be a major network of interest for such TRYCAT infusion experiments. Moreover, given the differences in the mechanism of action of 3-HK and QA, identification of the molecule contributing to the behavioural changes would have important implications regarding the understanding of CSD-induced brain pathology and the development of novel therapeutics. As stated above, there is currently diverse evidence for the cellular and molecular effects of 3-HK. Whilst 3-HK has been associated with free radical generation and induction of cell apoptosis, *in vitro* (Schwarcz and Pellicciari, 2002), it has also been proposed, based on *in vivo* evidence, that 3-HK acts more as a redox modulatory molecule than a neurotoxic metabolite (Colin-Gonzalez et al. (2014). In contrast, QA, with its NMDAR agonistic activity, is unequivocally recognised as a neurotoxic molecule (Schwarcz et al., 2012). Interestingly, 3-HK can interact synergistically with QA to potentiate the latter's effects on brain cells (Guidetti and Schwarcz, 1999), thus suggesting that both TRYCATs might act in unison to induce KYN pathway activation-dependent neuropathology.

Given our findings of CSD-induced DA deregulation and increased TRYCATs levels, studying 3-HK and QA effects on the DA system will be important with respect to attempting to identify the pathophysiological mechanism of stress-induced inflammation, brain pathology and behavioural disruption. As described above, 3-HK acts as a redox modulatory molecule while QA mainly acts via excitotoxicity and oxidative stress mechanisms (Colin-Gonzalez et al., 2014; Perez-De La Cruz et al., 2012). Of note, VTA DAergic neurons are under extensive control of glutamatergic transmission (Ungless et al., 2003) acting through NMDA receptors (Zweifel et al., 2011). Interestingly, Parkinson's disease (PD) research has shown that DA neurons are vulnerable to both excitotoxicity (Ambrosi et al., 2014) and oxidative stress (Blesa et al., 2015). Moreover, anti-oxidant compounds (e.g. N-acetylcysteine) and NMDAR antagonists (e.g. ifenprodil) provide neuroprotection to DA cells (Assous et al., 2014). This suggests that one of the mechanisms of action of the NMDAR antagonist ketamine as an antidepressant would be to counteract QA-induced excitotoxicity (Walker et al., 2013); at least in a subpopulation of patients with increased brain QA levels (Steiner et al., 2011). Thus, excitotoxicity and oxidative stress represent two 'drugable' molecular processes, blockade of which might counteract the relevant neuronal effects of stress-induced inflammation.

Another possibility is that TRYCATs might exert detrimental effects on DA transmission affecting dopaminoceptive cells. For example, QA increases Ca^{2+} influx and induces excitotoxicity in GABAergic striatal medium spiny neurons (MSNs), the major cell type in the NAcc (Perez-De La Cruz et al., 2012), and reduces striatal adenosine A2A and dopamine D2 receptors (Moresco et al., 2008). QA-induced Ca^{2+} influx provokes long-lasting dysregulation of the cytoskeletal homeostasis (Pierozan et al., 2014), and these effects are mediated by phosphorylating proteins, including PKA and Cdk5 (Pierozan et al., 2012). Activity of PKA and Cdk5 controls DARPP-32 phosphorylation status at Thr-34 and Thr-75 (see Introduction, section 2.1). DARPP-32 is enriched in striatal MSNs (Zachariou et al., 2006) and is a crucial mediator of the biochemical, electrophysiological, transcriptional, and behavioural effects of DA (Svenningsson et al., 2004). Therefore, QA might impact on post-synaptic DA signaling by modulating DARPP-32 activity, such that local synthesis of QA could disrupt dopaminoceptive cell function throughout the brain (Pierozan et al., 2014).

As presented above, excitotoxicity mechanisms represent a possible neuropathological pathway in depression (Steiner et al., 2012). However, the KYN pathway is certainly not the only potential candidate responsible for glutamate-dependent cell damage. Indeed, it has been proposed that Th17 cells could use glutamate-mediated excitotoxicity as a mechanism in the pathogenesis of multiple sclerosis (MS) (Kostic et al., 2014). Siffrin et al. (2010) have shown that Th17 cells induce Ca^{2+} changes in an antigen-independent manner in brain cells, thus inducing neuronal dysfunction. Interestingly, it has been shown that stress increases Th17 cells in the brain (Beurel et al., 2013), and I report increased Th17 cells in the spleen of CSD mice (Study B). However, I could not identify any effect of CSD on Th17 cells at the level of the whole brain. Histological analysis is needed to investigate whether a higher number of T cells is evident in the brain at different time points during CSD or if there might be a brain-region specific increase.

Studying the cellular and molecular effects of CSD on specific cell populations will be essential for a thorough understanding of the neuropathology associated with CSD-induced inflammation and behavioural changes, and for the identification of possible drug targets. As mentioned above, together with deregulation of the mesolimbic DA system, this laboratory has shown reduced expression of genes related to GABAergic interneurons (*Pvalb*) and oligodendrocytes (i.e. *Mag*, *Mobp*, *Opalin*, *Aspa*) in the amygdala (Azzinnari et al., 2014). However, despite the indication that CSD affects the GABAergic interneurons and oligodendrocytes, these data do not provide any information on the signaling pathways affected by CSD in those specific cell types. To answer this question, transgenic mice expressing fluorescent proteins (e.g. EGFP) controlled by cell-specific promoters (Lobo et al., 2006) can be used in combination with FACS to isolate DAergic neurons, GABAergic interneurons and oligodendrocytes. Analysing cell-type specific transcriptome changes using RNA-seq will be informative regarding the molecular events occurring in neurons and oligodendrocytes. Dysregulated genes and pathways will then need to be further validated *in vivo* using loss/gain-of-function experiments (Stuber and Mason, 2013), in order to identify those playing a causal role in the occurrence of CSD-induced behavioural disturbances. The ultimate goal of such an approach is to develop cell-specific pharmacotherapies targeting a defined dysfunctional gene product (Stuber and Mason, 2013). The advantage of cell-specific pharmacotherapies would be to overcome systemic toxicity and off-target effects, while enhancing therapeutic efficiency (Stuber and Mason, 2013). For example, pharmacological targeting of the KYN pathway using cell-specific IDO1 inhibition might be preferred to systemic administration (Sundaram et al., 2014). Indeed, despite the positive effect of systemic IDO1 inhibition in reducing the synthesis of QA within myeloid cells, it could also reduce the production of the neuroprotective KYNA in astrocytes, thus impairing the formation of neuroprotective metabolites within the brain (Sundaram et al., 2014). Interestingly, it

has been shown that systemic IDO inhibition can exacerbate clinical and histopathological parameters in the experimental autoimmune encephalomyelitis (EAE) model for multiple sclerosis (Sakurai et al., 2002). In preclinical animal research, cell type-specific transcriptional profiles associated to stress exposure as well as the targeting of specific gene products in a brain region- and cell type-specific manner, can be achieved using RNA-seq of FACS sorted cells and viral-mediated overexpression or knockdown of target genes to reverse the behavioural effect of stress (Stuber and Mason, 2013). However, the translational capability of such an approach to humans is limited. Thus, the development of biological tools allowing the assessment of cell-type specific status and cell-type specific targeting in humans is necessary (Stuber and Mason, 2013).

3. Implication for depression research

As I stated in the Introduction to my thesis, the clinical heterogeneity of depression is the phenomenological reflection of multiple pathophysiological routes targeting particular behavioural/cognitive domains (Webb et al., 2015). Resolution of such an heterogeneity and classification of mental disorders in term of dysfunction in specific behavioural domains is the objective of the NIHM RDoC initiative (Cuthbert, 2015). The RDoC project considers psychiatric diseases from a translational research perspective and supports the development of a precision medicine approach for mental disorders (Cuthbert and Insel, 2013). A precision medicine approach in psychiatry can be operationalized with studies that investigate the contribution of genetic, environmental and developmental factors to alteration of a defined behavioural dimension. However, behavioural assessment has to be coupled with the measurement of biomarkers to further boost the patient stratification and give precise indication for treatments. The link between the immune system and depression offers an example for such an approach. It has been shown that depression is associated with microglia activation in cortico-limbic brain regions, both in post-mortem studies (Torres-Platas et al., 2014) and *in vivo* using PET imaging (Setiawan et al., 2015). PET imaging provides brain region-specific information on microglia activation that can be used for patient-specific treatment indications. For example, administration of minocycline, which suppresses microglia activation (Seabrook et al., 2006), has been proposed as an adjunctive therapy for depression (Miyaoka et al., 2012; Soczynska et al., 2012). Thus, regarding the psycho-neuro-immune hypothesis for depression presented in this thesis, future human studies will have to investigate the existence of CNS inflammatory markers (e.g. TSPO PET imaging) and DA system dysregulation (e.g. fMRI brain activity during reward-related tasks) in homogeneous populations of patients sharing the alteration of a specific behavioural domain (e.g. reduced approach motivation). Preclinical investigations would focus on the same behavioural domain using the appropriate behavioural tests and investigate the effects of experimental manipulations (e.g. stress exposure) on brain inflammation and neurotransmission. Mechanistic experiments would then be informative on the causality existing between brain inflammation, neural transmission deregulation and behavioural disturbances, and would help identifying and validating novel treatment targets.

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